EXPERIMENTAL BONE MARROW REACTIONS.

III. Polycythemia, Normoblastosis, and Erythrocytic Hyperplasia of the Bone Marrow Produced by Gum Shellac.

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Plate 26.

(Received for publication, January 7, 1927.)

In the study, both experimental and clinical, of the hematopoietic system, attention has been directed almost exclusively to the peripheral blood. This is due to the ease with which the peripheral blood may be examined and the difficulties encountered when attempts are made to analyze the bone marrow and interpret the results obtained. The recent work of Sabin, Doan, and Cunningham (1–3), however, on the structure and function of the bone marrow has greatly facilitated the study of the hematopoietic organs. The theory of these authors as to the origin of red blood cells and clasmatocytes from the endothelium lining the sinuses of the bone marrow, furnishes a working hypothesis for the study of the function of the bone marrow, and its reaction to external influences.

In a series of investigations on bone marrow reactions the hypothesis of the endothelial origin of the red blood cells and the clasmatocytes has been used as a working basis. In a former study (4, *a*) it was found that a colloidal suspension of a heavy metal (collargol) produced an aplastic anemia, interpreted as due to injury to the endothelial cells of the hematopoietic organs. In a control experiment (4,*b*) with a colloidal suspension of carbon particles in the form of drawing ink, the results warranted the conclusion that carbon particles as such stimulate phagocytosis, but do not inhibit, to any appreciable extent, the formation of erythrocytes. This seems to indicate that the particles ingested by the phagocytic cells must exert a toxic action if they

are to suppress the function of the cells. The importance of this is evident in the choice of colloids for so called "blocking" of the "reticulo-endothelial system."

During the latter investigation with India ink, a non-dialysable substance passing through a Berkefeld filter was found to stimulate markedly the bone marrow as evidenced by the outpouring of nucleated red cells in the peripheral blood and erythropoietic hyperplasia of the bone marrow. Through the courtesy of F. Weber Co. the composition of this particular ink was obtained. All the ingredients were tried, but only one gave the results interpreted as a stimulation of the bone marrow. This was a gum shellac used as a protective colloid. Experiments were continued with this substance with two purposes in mind: first, to elucidate, if possible, the mechanism of stimulation of the red cells and their delivery, and secondly to see whether this gum would prove to be a therapeutic agent. The second project, in view of the many substances proposed and rejected as bone marrow stimulants, was entered upon with justifiable hesitation.

Most of the work on stimulation of the erythropoietic centers has been done in search for a therapeutic agent, the importance of which is evident and reflected in a vast literature.

No attempt will be made to review this as recent papers have summarized different phases of the question (5, 6, 7, *a*, 8). If, however, one accepts as criterion of erythropoietic activity the rise of the number of erythrocytes, together with the appearance of young cells in the peripheral blood stream and a hyperplastic marrow, the experimental evidences are meager. Polycythemia, due to various agents, is common. Many of those reviewed by Lamson (5) in 1915, however, may be explained in all probability by other conditions than actual bone marrow stimulation. Others give data insufficient for drawing any definite conclusions.

The recent search for therapeutic agents, stimulated by advances in our knowledge of the internal secretions and hormone activities, has been directed toward the isolation of substances in the animal organism. Following Danilewsky and Selensky (9), Krumbhaar and Musser (10) and Downs and Eddy (11) found that spleen extract caused a rise in the number of red blood cells with comparatively less increase in hemoglobin. Krumbhaar and Musser obtained no evidence of an increased number of young cells in the blood, while Eddy and Downs (7,*b*) found a gradually increasing normoblastosis in rabbits given large doses of protein-free splenic extract subcutaneously. The number of red cells in the peripheral blood fluctuated with a generally rising tendency. Neither of these authors report any examination of the bone marrow.

Leake and associates (12, *a*, *b*) found a combination of splenic and bone marrow extract more effective than splenic extract alone. In the rabbits injected the reticulated cells showed an increase in a few cases, while of six animals examined the bone marrow in two was hyperplastic. Whether this hyperplasia was erythropoietic is not stated.

The reports of Eddy and Downs (7, *b*) and Leake and Leake (12, *a*) seem to indicate that some definite effect was produced on the bone marrow. They conclude that hormones are involved. That some substance capable of stimulating red blood cell formation is present in the serum and the bone marrow after bleeding was suggested by Carnot and Deflandre (13).

Among inorganic agents for stimulation of the erythropoietic organs, iron and arsenic have been used extensively in clinical work. Experimental work seems to indicate that neither iron nor arsenic exerts any marked effect on blood formation (14–16). Nissen (17), however, reports that erythroblastic hyperplasia is produced by the intravenous injection of "elektroferrol." Excluding anemia from various causes, and especially anemia due to blood loss, low oxygen tension (18, 19) seems to be the most effective stimulant of blood formation as evidenced by increase in erythrocytes and a hyperplastic marrow.

Material and Methods.

The gum shellac employed was obtained from the F. Weber Co., Philadelphia. Gum shellac is said to be a product of plant excretion or secretion, due to the action of the lac insect, *Coccus lacca*. Chemically it has been partly analyzed although some of its components are very unstable (20). The gum shellac used is soluble in water containing sodium borate. In concentrated solutions it is a cloudy dark brown fluid which in dilutions employed after filtering appears as a clear, port wine-colored liquid. The solutions employed varied in strength from 4.4 to 0.44 per cent of the gum, and, correspondingly, the salt used to keep the gum in solution varied from 1.8 to 0.18 per cent. The solutions were sterilized in the autoclave, occasionally by boiling. Injections varied both in amount and interval of administration, as it was desirable to see the effects not only of various concentrations but also of the frequency of administration. The experimental procedure was essentially the same as in a previous investigation (4, *b*).

Bone marrows were studied from three points of view: in sections, in smears made directly from the bone marrow, and in smears made after centrifuging the emulsified bone marrow in 2 per cent sodium citrate. In addition, supravital counts were made of all the bone marrows, following the method of Sabin (3, *b*).

Rabbits bought in the market usually improve when given better food in the laboratory. To exclude this factor as far as possible, they were kept in the stock pen for a considerable period, then put in individual cages under the same conditions for from 2 to 4 weeks before the investigation was begun.

EXPERIMENTAL.

Healthy male rabbits were used except in a few instances which will be noted. They were divided into groups according to the strength of the solution employed: (1) Strong solution—equal to the amount of gum shellac contained in the original ink; *i.e.*, 4.4 per cent. This was intensely toxic. (2) Medium solution—2.2 per cent. This was toxic to some animals, stimulating to others, as evidenced by increase of red blood cells and hemoglobin and a characteristic outpouring of nucleated red cells. (3) Weak solution—1.1 per cent. This was usually stimulating, although slightly so in some cases. In 0.4 per cent solutions no reaction was elicited.

I. Injections of 4.4 Per Cent Gum Shellac Solution.

Nine animals were injected with a 4.4 per cent solution. Of these five died instantaneously after the first intravenous injection of 4 cc., two died suddenly after the second dose, and one after four doses of 2.5 cc., and a fifth dose of 3 cc. The remaining animal had seven doses of 4 cc., subcutaneously, without apparent systemic effects. Locally there was induration and edema of the subcutaneous tissues but no apparent necrosis.

The mode of death in the animals was similar. About ½ minute after the injection, and in a few instances before the needle had been withdrawn, there were signs of collapse with rapid breathing but little or no muscular contraction of body and limbs. Then the breathing stopped while the heart continued to beat for a short varying interval after the cessation of respiration. At autopsy the lungs were found collapsed with little or no injection; the right heart was distended and engorged with blood; the left heart was empty; the abdominal viscera were greatly congested. The impression was gained that there was a sudden interruption of the blood flow in the pulmonary circulation. The microscopical sections gave no clue to the cause of death. Many of the smaller and medium sized vessels were closely packed with erythrocytes but no agglutination of the latter had taken place as far as could be determined from fixed sections.

In conjunction with Dr. V. H. Norris, the effect of the solution on blood pressure and respiration was tested. With a fatal dose in an

animal under heavy morphine anesthesia the respiratory rate increased and the blood pressure rose, but the animal had convulsions in the agonal struggle thus interrupting the record. When smaller, non-fatal doses were given, there was a definite increase in the respiratory rate as well as a rise in blood pressure, both well sustained for a considerable period of time. Physiological sodium chloride in the same dosage had no effect on respiration or blood pressure.

The cause of sudden death in animals from toxic substances as well as death due to anaphylactoid phenomena has been analyzed by many observers (21–23). It is probable that the fatal outcome in the animals injected with 4.4 per cent gum solution may be explained as an anaphylactoid phenomenon, although paralysis of the respiratory center cannot be excluded. The fact stands out that a solution of this strength given intravenously was fatally toxic to all the animals injected. This is in sharp contrast to the effect of the same substance in contact with carbon particles in the India ink which in no instance was fatal in the same concentration. The inert carbon particles must have exerted a detoxifying influence on the solution in which they were suspended. That they acted as a protective was shown in one animal which had been injected with India ink to which had been added an equal quantity of the 4.4 per cent solution of gum shellac. Three injections of 4 cc. each were given without ill effect. The fourth injection of 4 cc. of the solution in which the ink had been replaced by distilled water caused the death of the animal in the same sudden manner as described above.

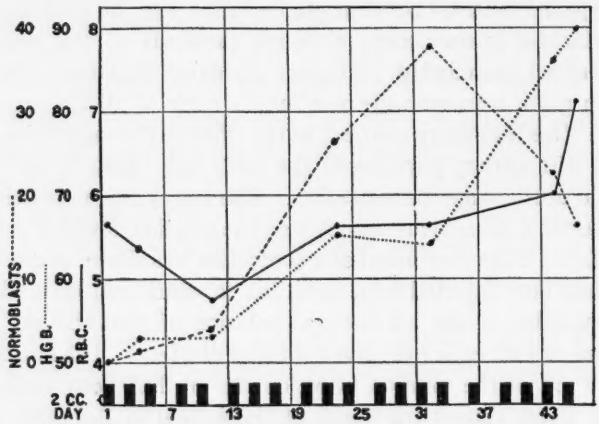
II. Injection of 2.2 Per Cent Gum Shellac Solution.

Seventeen rabbits were injected with 2.2 per cent gum shellac solution. Of these four died as a result of the injection. Three died suddenly, one after the first, one after the second, and one after the fifth dose. The remaining animal was found dead 24 hours after the second dose.

Microscopically, No. H-76, succumbing after the first dose, showed extensive hemorrhage in the bone marrow, amounting practically to a dissolution of the vascular bed. This animal had indications of a hyperplastic marrow prior to the injection caused by loss of blood. No. H-91, dying after the fifth injection, showed a hyperplastic bone marrow, comprising mainly the erythrocytic series, with a

moderate number of megaloblasts and erythroblasts and a great many normoblasts. Endothelium, wherever seen, was hypertrophied. In addition, the megalocaryocytes were increased and an occasional clasmatocyte was seen filled with a yellowish pink substance which did not take the iron stain. No pathology to account for the sudden death could be made out in any of the animals except in No. H-95 which died 24 hours after the second dose, showing fibrinous exudate in the lung alveoli.

Thirteen rabbits were repeatedly injected with a 2.2 per cent gum shellac solution. All these animals showed one thing in common; namely, the outpouring of normoblasts and later erythroblasts in the



In Charts 1 to 5 the number of normoblasts is in thousands, the hemoglobin in per cent, and the erythrocytes in millions.

CHART 1. Rabbit H-83 illustrates the rise of hemoglobin and erythrocytes and the outpouring of normoblasts after medium sized doses in an animal with achromia of the red blood cells. The bone marrow showed intense hyperplasia.

peripheral blood. The number of red blood cells and the hemoglobin values following the injection of this strength solution were variable, some animals showing a slight initial drop after several doses, followed by a rise, others an initial rise followed by a decrease. The lowest point of erythrocytes and hemoglobin was usually concomitant with the greater number of nucleated red blood cells in the peripheral blood stream, although exceptions occurred.

Rabbit H-83, Chart 1, when injections were begun, was in good condition except for low hemoglobin and a marked achromia of the erythrocytes. Twenty 4 cc. injections were administered over a period of 45 days. The first few doses were followed by a slight fall in the number of erythrocytes, concomitant with a slight increase in white blood cells; then there was a definite rise, both in hemoglobin and erythrocytes. Nucleated red blood cells were present in the peripheral blood after the second dose, the highest number (37,600 per c.mm.) being reached on the 32nd day, after which there was a decline with still further increase in hemoglobin and erythrocytes. The animal was killed at a time when the hemoglobin showed an increase of 80 per cent of the initial determination and the red cells an increase of approximately 36 per cent. The general condition was excellent, the weight had

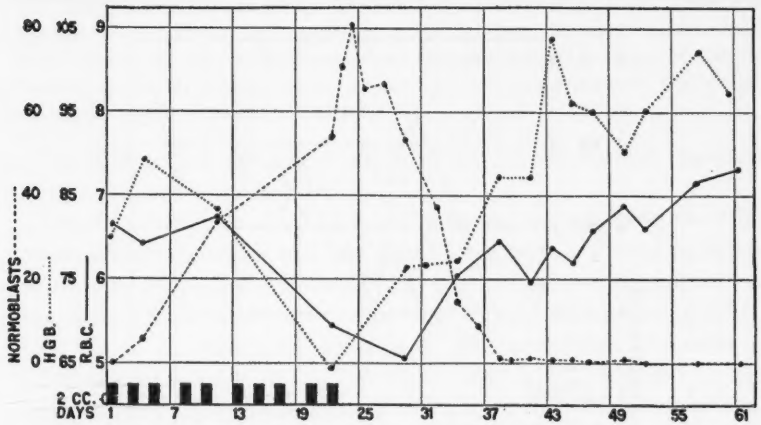


CHART 2. Rabbit H-84 showed a tremendous number of nucleated red cells in the peripheral blood stream, which did not disappear until the 28th day after the last injection. Bone marrow 38 days after last dose was essentially normal.

increased from 2610 to 2775 gm. The gross autopsy findings were normal except for an enlarged spleen and liver. Microscopically, the liver, spleen, and bone marrow showed a great many phagocytic cells, enlarged, staining a pale pink with eosin, and containing various sized conglomerations of a substance staining a deep red. The spleen contained many nucleated red cells, mainly normoblasts. Many open spaces were lined with cells which looked like erythroblasts and megaloblasts, and the impression was gained that erythrocyte hyperplasia was present in the spleen. The protoplasm of the liver cells looked vacuolated and granular, but as this was observed in untreated animals it probably may be disregarded. The kidney showed a few patches of fibrosis in the medullary region with occasional round cell infiltration in the cortex. There was no indication of increased blood destruction, no iron being found. The bone marrow was intensely hyperplastic.

This hyperplasia included mainly the erythrocyte series and the megalocaryocytes. The normoblasts and late erythroblasts predominated but a considerable number of the earlier forms were also present (see Fig. 1.) The predominance of the nucleated red cells was verified additionally by (1) supravital studies, (2) fixed smears prepared directly from the bone narrow, and (3) fixed smears prepared after centrifuging the emulsified bone marrow in 2 per cent sodium citrate. From these additional studies it was found that the leucocytic elements constituted about 25 to 38 per cent and the nucleated red cells from 62 to 75 per cent, varying with the method used, but giving an average value of one-third white blood cells and two-thirds nucleated red cells. That the increase was considerable may be concluded from the fact that most of the fat cells of the bone marrow had been replaced, although some deduction of space must be made for the clasmato-

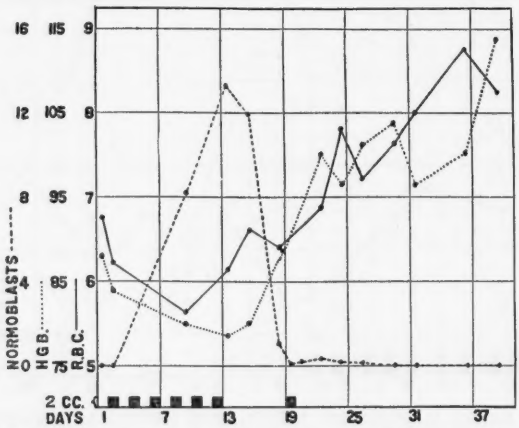


CHART 3. Rabbit H-100 illustrates the effect of small dosage. Normoblastosis was less striking, but the final rise in erythrocytes and hemoglobin was marked.

cytes which were both enlarged and increased in number. The blood vessels of the bone marrow were everywhere intact but the high power lens frequently showed these vessels to be lined with nucleated red cells in various stages of maturation (see Fig. 2.)

To see how long the presence of nucleated red blood cells would continue in the peripheral blood, Rabbit H-84 was given 10 doses of a 2.2 per cent solution (see Chart 2.) On the 2nd day after the last dose 82,400 erythroblasts and normoblasts per c.mm. were found in the blood stream. Not until the 28th day after the last injection did the nucleated red blood cells disappear permanently from the peripheral blood stream. The stimulation in this animal seemed to produce a tremendous excess of cells of the erythrocyte series, and the subsequent course indicated that these cells gradually matured and were thrown into the peripheral

circulation, increasing the hemoglobin and the red cells. At autopsy, 38 days after the last dose, little or no evidence was present in the bone marrow to indicate the stage of intense erythropoietic hyperplasia through which the animal had passed.

Rabbit H-100, Chart 3, illustrates the effect of smaller dosage (2 cc.). The initial decrease of red blood cells and hemoglobin was negligible, the outpouring of the nucleated red cells was correspondingly less, the final rise of hemoglobin and erythrocytes was marked, with values customarily not encountered in rabbits. There was an increase in weight from 2755 to 3160 gm., and when killed the animal was in perfect condition except for an enlarged but normal looking liver and kidney. Microscopically, the bone marrow was normal except for slight irregularity in the contour of the fat cells. By estimation the proportion of cells seemed normal; *i.e.*, about two-thirds leucocytes, one-third of the erythrocyte series. This was verified by fixed smears and supravital studies in which the actual counts varied from 33 to 37 per cent nucleated red blood cells, and from 63 to 69 per cent white blood cells. A few clasmatocytes were enlarged in the bone marrow; the number of megalocaryocytes was not increased.

Another animal treated similarly to No. H-100 showed the same results somewhat less marked.

In control animals the cells in the bone marrow averaged one-third nucleated erythrocytes and two-thirds leucocytes. This is in agreement with Itami (24) who found that the nucleated red bood cells in the normal rabbit's marrow constituted 29½ per cent, excluding primitive cells. In comparing animals which were killed immediately after discontinuation of the injections with animals allowed to live for a variable time, the impression was gained that the enlarged clasmatocytes had decreased in number in those allowed to live, thus indicating that an elimination of their contents was taking place.

III. Injection of 1.1 Per Cent Gum Shellac Solution.

Twelve rabbits were injected with 1.1 per cent solution. Both dosage and time interval between injections were varied to see whether optimum effects could be obtained.

With 4 cc. doses given at the same intervals as No. H-100, Chart 3, the results were practically the same as with 2 cc. of a 2.2 per cent solution.

The effect of 4 cc. doses administered at longer intervals is illustrated in Chart 4, Rabbit H-104. At the beginning of the investigation this animal was thin, ill

looking, anemic, with 2,970,000 red blood cells and 52 per cent hemoglobin. No cause for the anemia was discovered; the animal had been isolated for some time and no external injury or blood loss could be found. The peripheral blood findings remained stationary for about 1 week except for the day on which the first injection was made when the reticulated count had risen to 9.5 per cent. The response was prompt. 3 hours after the injection the reticulated count rose to 15.3 per cent, with 1200 nucleated red blood cells per c.mm. Nucleated red blood cells were present in the blood stream up to the 4th day as a result of the first injection. Another injection 4 days after the first dose caused a still greater outpouring of normoblasts. At this time the reticulated count was still high; namely, 13.2 per cent. Before the third injection was given all the normoblasts

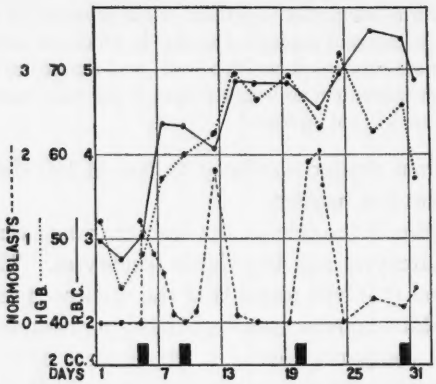


CHART 4. Rabbit H-104. Thin, anemic animal given 4 cc. doses of 1.1 per cent gum shellac solution at long intervals. The prompt response to each injection is noteworthy. Final hemorrhages in the glomeruli of the kidneys.

had disappeared from the peripheral blood and the reticulated count was approximately normal (2.6 per cent). The prompt response to the third injection is indicated on the chart. In 26 days the blood count had reached a low normal value and the animal was gaining weight, although far from appearing healthy. The fourth injection was given 10 days later. Next morning the rabbit was moribund. At autopsy the large kidneys, with extensive hemorrhages throughout, were noteworthy. Microscopically, this was verified, practically every glomerulus being obliterated. There were numerous hemorrhages in the lung and around the central vein of the liver. The bone marrow was hyperplastic. This hyperplasia included mainly the leucocytic series. There were no evidences of dissolution of the vascular bed of the bone marrow.

The outstanding features in Rabbit H-104 were (1) the prompt response to the injections, evidenced by the increase in reticulated cells and nucleated red blood cells and the rise in the number of erythrocytes and hemoglobin, and (2) the final hemorrhages. Possibly the kidneys were damaged before injections were begun, but there seems to to be no doubt that the last injection was the cause of the extensive hemorrhages.

The striking similarity in the picture of blood regeneration after hemorrhage and the appearance of normoblasts in the peripheral blood after injection of gum shellac solution is well illustrated in No. H-104 and healthy companion animals. In No. H-104 a few normoblasts were present in the blood 3 hours after the first injection, while in healthy animals they did not appear till after 24 hours. After hemorrhage in rabbits nucleated red blood cells appear in 24 to 48 hours (25–27). Usually the normoblastic crisis after hemorrhage is of short duration, while after gum shellac injection the presence of nucleated red blood cells in the peripheral blood stream was more prolonged.

A healthy rabbit treated in the same way as No. H-104 did not show any signs of illness after the fourth dose. To see whether hemorrhage could be produced, 4 cc. of a 2.2 per cent solution (double previous doses) was given 6 days after the fourth dose. When killed 24 hours later a few small hemorrhages were found in the lungs and in the bone marrow. The latter had been suspected from the appearance of the peripheral blood. The white blood cells had risen from 14,000 to 98,000 cells per c.mm. and many immature forms were present.

As hemorrhages had not been a feature of the previous experiments in which the injections had been given every 2nd day, they seemed to be the result of injections after long intervals apparently after the animal had been sensitized to the drug. To test this theory, one animal was injected in the same manner as No. H-84 (see Chart 2). 40 days after the administration of the last consecutive dose the equivalent of a single previous dose was given. When killed the following day, considerable hemorrhages were found in the bone marrow, corroborated before death by the appearance of immature cells of both the

leucocyte and erythrocyte series in the peripheral blood. Rabbit H-102 was given six doses of 4 cc. each of the 1.1 solution. 16 days later another dose produced no ill effect. Hemorrhages in the bone marrow were found, however, after a similar dose administered 14 days later. In this animal the surface of the kidney was studded with what appeared to be hemorrhagic spots. The microscopical examination revealed this to be an extreme dilatation of the blood vessels in about 50 per cent of the glomeruli, amounting to dissolution of the vascular bed in a few instances. The bladder urine showed blood microscopically.

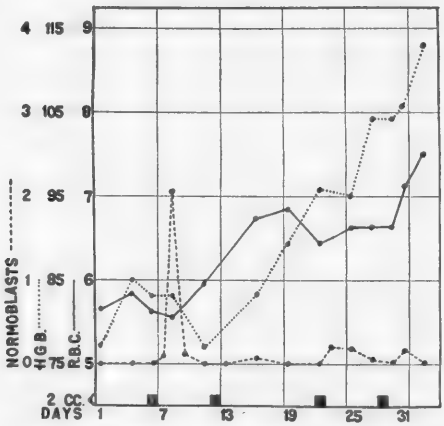


CHART 5. Rabbit H-105. Healthy animal given 2 cc. doses of 1.1 per cent gum shellac solution at long intervals. Normoblastosis present, but slight. No hemorrhages produced.

It is to be noted that in all these animals showing hemorrhages, the red blood cells and the hemoglobin had risen above the initial level. The fact remains, however, that the administration of 4 cc. doses of the 1.1 per cent solution at long intervals was toxic to the endothelium of the sinuses of the bone marrow and the vessels of the glomeruli after a certain time interval. With the same dosage as above (4 cc. of 1.1 per cent solution) given in divided doses at hourly intervals once a week, no ill effects were observed.

The effect of smaller dosage of the 1.1 per cent solution is illustrated in Chart 5, Rabbit H-105.

Four 2 cc. doses were given over a period of 26 days. At the end of the experiment the animal was in excellent condition. The bone marrow was moderately hyperplastic in structure. Leucocytic hyperplasia predominated, although at the edges of the cross-section there was considerable erythrocytic hyperplasia, the cells being mainly in the normoblastic stage. Blood vessels were intact, wide open, lined with a swollen endothelium wherever endothelium could be seen, otherwise with nucleated red blood cells in all stages of development.

In conjunction with Mr. Ralph Wheeler the effect of a certain quantity of gum shellac given in a definite time was obtained. As far as possible the conditions were kept constant, the variables being the time interval and the size of the dose. Medium sized doses given every 2nd day were found to be more effective than larger doses twice a week or smaller doses every day.

DISCUSSION.

From the findings described above, it is evident that gum shellac is an agent which markedly influences the hematopoietic organs. In common with many drugs this substance was fatally toxic in strong doses, and without any demonstrable effect in minimal doses. Medium strength solutions produced normoblastosis, accompanied by a period of slight decrease of red blood cells and hemoglobin, concomitant with the greatest outpouring of nucleated red blood cells. This normoblastosis was either preceded or followed by a rise in erythrocytes and hemoglobin above the initial levels which occurred during the period of injections or as soon as the injections were discontinued. Microscopically, an erythrocytic hyperplasia of the bone marrow was found immediately after discontinuation of the injections, and there was a return to normal conditions after variable intervals of time. With small doses the nucleated red blood cells in the peripheral blood stream were numerically less; the decline in erythrocytes and hemoglobin was minimal or altogether absent; the rise of erythrocytes and hemoglobin during and after the administration was, in many instances, greater than with medium sized doses. Hemorrhages,

mainly in the bone marrow and occasionally in the kidney glomeruli, were obtained under two sets of circumstances: (1) With a fatal dose in one animal which prior to injection apparently had a hyperplastic bone marrow. (2) *a*, After the fourth or fifth medium sized dose administered at intervals of from 4 to 19 days, and *b*, after an injection following a rest period preceded by a course of continuous injections. The latter phenomena indicated some kind of sensitization of the vascular endothelium. A precipitin reaction with gum shellac was obtained both with normal serum and serum from animals sensitized with gum shellac solution.

The explanation of the stimulation produced must be sought either in an increased blood destruction and compensatory regeneration, or in a direct stimulation of the bone marrow.

The concept suggested by Morawitz (28) that products of red blood cell destruction may normally stimulate the bone marrow is an attractive one, but it does not explain blood regeneration after hemorrhage. Some authors (7, 11) have suggested that splenic extract increases the blood destruction, a hormone being formed from these broken down cells capable of stimulating the bone marrow. Small doses of hemolytic serum subcutaneously (29), arsenic (30), and toluylenediamine (31), both hemolytic toxins, produce an increase of erythrocytes. Blood regeneration after destruction of red blood cells by phenylhydrazin is more rapid than after hemorrhage (32).

The evidences of blood destruction after injection of gum shellac are meager. Animals examined at frequent intervals for the first 24 hours after injection showed no decline but rather a rise in erythrocytes and hemoglobin. After several injections there was a decline in red blood cells and hemoglobin, concomitant with the presence of a great number of nucleated red blood cells in the peripheral blood, but this decline did not amount to an actual anemia which would explain the marked increase in the production of erythrocytes. The smears showed no undue increase of fragmentation. With the method used no appreciable increase of iron was found in the tissues. Hemoglobinuria was not observed. The concept of an appreciably increased blood destruction after injections of gum shellac solution can, therefore, be dismissed at least tentatively.

As evidences of stimulation of the bone marrow have been primarily

sought in the peripheral blood without examination of the bone marrow the facts reported in the literature are difficult to evaluate. Excluding anemia, erythrocytic hyperplasia has been produced by low oxygen tension (18, 19), and various colloids, such as "elektroferrol" (17) and collargol (4, *a*, 33). In this connection it may be of interest to note that Metchnikoff in 1900 (34) observed the relation between increased phagocytosis and new formation of red blood cells in lepers injected with horse serum.

The evidences for direct stimulation of the bone marrow in the present investigation are suggestive although not conclusive. The substance injected was taken up, at least partly, by the phagocytic cells in the hematopoietic organs. Whether this substance, visible in the phagocytic cell, is the actual stimulant of red blood cell formation or an accessory cannot be determined from data in hand. That the endothelial cells are strongly affected is evidenced by their hypertrophy and the hemorrhages, produced by a fatal dose in one animal with a hyperplastic marrow and after the administration of the drug at long intervals in others. It is of interest to note that the hemorrhages produced occurred in all instances after erythropoietic stimulation. The response varied with the state of activity of the endothelial cells; *i.e.*, an inactive cell was stimulated, while a hyperactive cell responded to the same dosage with overstimulation which brought about complete permeability or cell death.

Gum shellac seems to be a substance which stimulates the blood-forming organs to a higher degree than any other substance heretofore reported. Its complexity, toxicity, and the possible necessity for intravenous administration renders therapeutic application as yet undesirable. The effect of this drug on the endothelium may throw some light on the various reactions of the hematopoietic system.

CONCLUSIONS.

1. The effect produced by intravenous administration of gum shellac solution varied directly with the strength of the solution and the amount injected.

(*a*) Strong solutions were intensely toxic and fatal to all the animals injected. Toxicity was abolished by the presence of carbon particles in the solution.

(*b*) Medium strength solutions produced a marked normoblastosis followed by an erythroblastosis if injections were continued, accompanied either by an initial rise of red blood cells and hemoglobin followed by a decrease or an initial decline followed by an increase. The decrease of erythrocytes and hemoglobin did not amount to an actual anemia, and was usually concomitant with the greatest outpouring of nucleated red cells.

(*c*) Small doses elicited the same kind of response except that the normoblastosis was less marked, the decrease of red cells and hemoglobin minimal or absent, the increase of red blood cells and hemoglobin marked in most instances.

(*d*) Minimal doses elicited no appreciable reaction.

2. The bone marrow in animals killed after a course of injections showed intense erythrocytic hyperplasia. This disappeared after a variable length of time with a return of the bone marrow to apparently normal condition.

3. Toxic effects with non-fatal doses in form of hemorrhages were produced mainly in the bone marrow and occasionally in the kidney under the following conditions.

(*a*) After one injection in an animal with hyperplastic marrow (hemorrhages in the bone marrow only).

(*b*) After the fourth and fifth dose when administered at long intervals (4 to 19 day intervals).

(*c*) After one injection given 40 days after several successive injections (one animal only).

4. Small and divided doses administered at long intervals produced no apparent ill effect.

5. Subcutaneous administrations did not elicit any marked systemic reactions. Locally there was induration and edema.

6. The conclusion was drawn that gum shellac solution stimulates markedly the production of erythrocytes in the bone marrow. The mechanism of stimulation is not clear, but it seems unlikely that it is due to destruction of red blood cells in the peripheral blood stream.

BIBLIOGRAPHY.

1. Doan, C. A., Cunningham, R. S., and Sabin, F. R., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.

2. Cunningham, R. S., Sabin, F. R., and Doan, C. A., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 227.
3.(*a*) Sabin, F. R., Doan, C. A., and Cunningham, R. S., *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 125. (*b*) Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 277.
4.(*a*) Muller, G. L., *J. Exp. Med.*, 1926, xliii, 533, (*b*) *J. Exp. Med.*, 1927, xlv, 399.
5. Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1915, vii, 169.
6. Drinker, C. K., Oxford Medicine, Oxford University Press, New York, 1920, ii, 509.
7.(*a*) Eddy, N. B., *Endocrinology*, 1921, v, 461. (*b*) Eddy, N. B., and Downs, A. W., *Am. J. Physiol.*, 1922–23, lxiii, 479.
8. Leake, C. D., and Evans, J. S., *Am. J. Med. Sc.*, 1924, clxviii, 819.
9. Danilewsky, B., and Selensky M., *Arch. ges. Physiol.*, 1895, lxi, 264.
10. Krumbhaar, E. B., and Musser, J. H., Jr., *J. Exp. Med.*, 1914, xx, 108.
11. Downs, A. W., and Eddy, N. B., *Am. J. Physiol.*, 1922, lxii, 242.
12.(*a*) Leake, C. D., and Leake, E. W., *J. Pharmacol. and Exp. Therap.*, 1923–24, xxii, 75. (*b*) Leake, C. D., and Bacon, F. J., *J. Pharmacol. and Exp. Therap.*, 1924, xxiii, 353.
13. Carnot, P., and Deflandre, C., *Compt. rend. Acad.*, 1906, cxliii, 384, 432.
14. Hooper, C. W., Robscheit, F. S., and Whipple, G. H., *Am. J. Physiol.*, 1920, liii, 263.
15. Whipple, G. H., and Robscheit, F. S., *Arch. Int. Med.*, 1921, xxvii, 591.
16. Musser, J. H., Jr., *Arch. Int. Med.*, 1921, xxviii, 638.
17. Nissen, R., *Z. ges. exp. Med.*, 1922, xxviii, 193.
18. Schauman, O., and Rosenqvist, E., *Z. klin. Med.*, 1898, xxxv, 126, 315.
19. Dalwig, H. C., Kolls, A. C., and Lowenhart, A. S., *Am. J. Physiol.*, 1915, xxxix, 77.
20. Dieterich, K., Analysis of resins, balsams and gum-resins, Scott, Greenwood and Co., London, 1901.
21. Coca, A. F., *J. Immunol.*, 1919, iv, 219.
22. Hanzlik, P. J., and Karsner, H. T., *J. Pharmacol. and Exp. Therap.*, 1919–20, xiv, 379, 425, 449, 479.
23. Manwaring, W. H., and Marino, H. D., *J. Immunol.*, 1923, viii, 317.
24. Itami, S., *Folia hæmatol.*, 1908, vi, 425.
25. Koeppe, H., *Münch. med. Woch.*, 1895, xlii, 904.
26. Zenoni, C., *Virchows Arch. path. Anat.*, 1895 cxxxix, 185.
27. Bunting, C. H., *J. Exp. Med.*, 1906, viii, 625.
28. Morawitz, P., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xi, 277.
29. Cantacuzene, J., *Ann. Inst. Pasteur*, 1900, xiv, 378.
30. Schustroff, N., and Wlados, Ch., *Folia hæmatol.*, 1923, xxix, 99.
31. Hertz, R., and Erlich, M., *Deutsch. Arch. klin. Med.*, 1914, cxvi, 43.
32. Blumenthal, R., and Morawitz, P., *Deutsch. Arch. klin. Med.*, 1907–08, xcii, 25.
33. Ribadeau-Dumas, L., and Debré, R., *Compt. rend. Soc. biol.*, 1908, lxv, 289.
34. Metchnikoff, E., and Besredka, A., *Ann. Inst. Pasteur*, 1900, xiv, 402.

EXPLANATION OF PLATE 26.

FIG. 1. Rabbit H-83. General appearance of bone marrow immediately after discontinuation of injections of gum shellac solution showing marked erythrocytic hyperplasia. × about 685.

FIG. 2. Rabbit H-114. Shows a blood vessel lined with nucleated red cells in various stages of maturation after a short course of injections of 1.1 per cent gum shellac solution. × about 820.

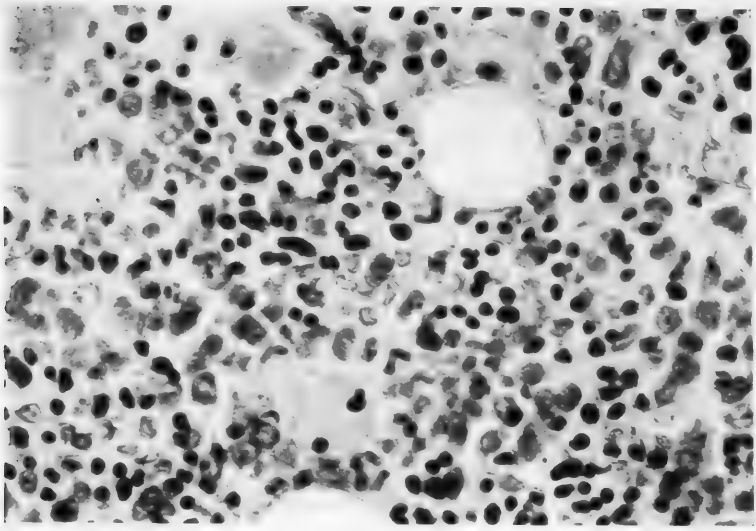


FIG. 1.

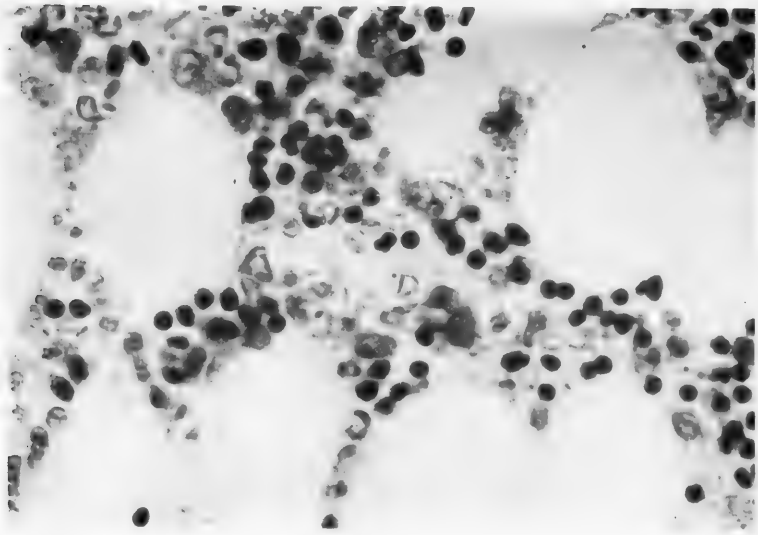


FIG. 2.

(Muller: Experimental bone marrow reactions. III.)

THE EFFECT OF GUM SHELLAC SOLUTION ON THE SURFACE TENSION OF RABBIT SERUM.

By J. M. HAYMAN, Jr.*

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(Received for publication, January 7, 1927.)

One of the interesting points in the action of the gum shellac solution studied by Muller (1) is the short interval after a single intravenous injection in a rabbit at which changes in the peripheral blood picture may be detected. The reaction rapidly subsides, and within a few days the blood picture has returned to normal.

It seemed possible that one factor in this series of events might be the changes in the physicochemical equilibrium of the plasma produced by the gum shellac solution which facilitated the removal of cells from the marrow sinuses. Among such changes, any which resulted in alterations of the properties of cell-serum interface might be most important.

The existence of such changes has not been determined directly. An indication of changes in the physicochemical state of the plasma after injection of this gum in rabbits was sought in measurements of the surface tension of the serum.

Du Noüy (2) has shown that the most satisfactory method of studying the surface tension of serum consists in measurements by the du Noüy tensiometer of the time-drop in diluted sera; that is, the difference between the initial surface tension of the diluted serum and that after equilibrium is reached. Since blood serum contains substances which lower surface tension, when pure serum is exposed to air the surface energy of its free surface decreases progressively for about 20 minutes. When serum is diluted with physiological salt solution the decrease of surface energy is less rapid, equilibrium being reached in approximately 2 hours. In a study of the changes in the surface

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TABLE I.

Surface Tension of Dilutions of Rabbit Serum after Intravenous Injection of Gum Shellac Solution.

Rabbit No.	Dilution of serum	Before injection			After injection of gum											
					½ hour			24 hours			48 hours			144 hours		
		Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop
		dynes	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*
7	1: 5,000	74.4	65.3	9.1	74.3	60.5	13.8									
	1:10,000	75.5	67.6	7.9	75.1	61.4	13.7									
	1:15,000	75.5	71.2	4.3	75.5	64.3	11.2									
9	1: 5,000	72.8	64.5	8.3	75.4	61.1	14.3				75.4	64.7	10.7			
	1:10,000	73.8	65.5	8.3	75.6	63.4	12.2				75.6	66.2	9.4			
	1:15,000	74.5	68.2	6.3	75.8	64.6	11.2				75.8	68.8	7.0			
12	1: 5,000	70.2	64.8	5.4				75.5	62.5	13.0						
	1:10,000	72.8	66.2	6.6				75.3	64.6	10.7						
	1:15,000	74.3	67.6	6.7				75.5	65.4	10.1						
3	1: 5,000	72.3	63.2	9.1				76.4	64.5	11.9	76.2	61.2	15.0	75.3	76.0	8.3
	1:10,000	76.2	65.7	10.5				76.5	67.0	9.5	76.5	62.5	14.0	76.6	67.6	9.0
	1:15,000	76.2	68.1	8.1				76.5	65.2	11.3	76.4	65.7	10.7	76.6	70.6	6.0
5	1: 5,000	75.3	67.0	8.3							75.1	60.2	14.9	72.8	64.5	8.3
	1:10,000	76.6	67.6	9.0							76.2	61.1	15.1	73.8	65.5	8.3
	1:15,000	76.6	70.6	6.0							75.4	60.5	14.9	74.5	68.2	6.3

11	1: 5,000	73.3	64.1	9.2								75.6	66.4	9.2		
	1:10,000	75.1	65.4	9.7								75.8	67.0	8.8		
	1:15,000	75.3	66.2	9.1								75.8	68.0	7.8		
15	1: 5,000	73.0	65.5	7.5				72.0	56.0	16.0						
	1:10,000	74.4	68.7	5.7				76.4	61.0	15.4						
	1:15,000	75.2	68.6	6.6				76.1	63.4	12.7						
16	1: 5,000	73.4	62.5	10.6				76.0	60.2	15.8						
	1:10,000	74.5	65.7	8.8				76.2	60.4	15.8						
	1:15,000	74.3	66.8	7.5				76.3	64.3	12.0						

tension of rabbit serum which accompany immunization, du Noüy (3) adopted a technique which involved the dilution of the serum to 1:10,000, a volume of 2 cc. of liquid, watch-glasses giving a diameter of about 4 cm. of free liquid surface with this volume of fluid, and measurement of the decrease in surface tension, or time-drop, in 2 hours. Surface tension was measured by the du Noüy tensiometer.

Methods.

We have followed du Noüy's technique. Great care was taken to have all glass with which serum, its dilutions, or distilled water came in contact strictly clean. Glassware was cleaned by boiling in sulfuric acid—dichromate mixture. Watch-glasses were usually washed and used on the same day; occasionally watch-glasses were used which had been washed the preceding day. All water was glass-distilled. Salt solution (0.9 per cent) used for diluting serum was made from Kahlbaum's NaCl, and when evaporated in a clean watch-glass gave crystals similar to those pictured by du Noüy (4). The surface tension of the salt solution was repeatedly checked, and did not show any fall in 2 hours.

Only rabbits having a normal time-drop were used (3). Blood was obtained by cardiac puncture. 5 cc. of a 1.1 per cent gum shellac solution were injected into an ear vein, and blood obtained after intervals of 30 minutes, 24, 48, and 144 hours. We are indebted to Dr. Muller for a supply of the gum shellac solution which she used. In most experiments a second rabbit was bled at the same time and at the same intervals as the experimental animal. This control served the double purpose of showing the effect of repeated bleedings of 3 cc. on the surface tension of the serum, and of showing that observed changes in the time-drop were not due to chance variations in room temperature, in the particular batch of salt solution used, or to other technical errors.

Three dilutions of serum, 1:5,000, 1:10,000, and 1:15,000 were made from the serum obtained after centrifugation. The serum was diluted 1:100 in volumetric flasks, and further dilutions made in test-tubes. Pipettes were not recalibrated. These three dilutions, rather close together, were chosen not only as a check on the accuracy of the technical manipulations, but because it seemed possible that if the gum shellac solution had any demonstrable effect on the surface tension of serum, it might be manifested by a change in the concentration at which the greatest time-drop occurred. With the technique used du Noüy has found that the maximum time-drop with normal serum takes place around 1:10,000 dilution.

The watch-glasses were brought under the tensiometer ring by means of the turntable described by du Noüy. For the use of this apparatus and for watch-glasses of approximately the proper curvature, I am indebted to the great kindness of Dr. du Noüy's laboratory. Care was taken to increase the torsion on the tensiometer wire uniformly and slowly. The room temperature was maintained as close to 23°C. as possible.

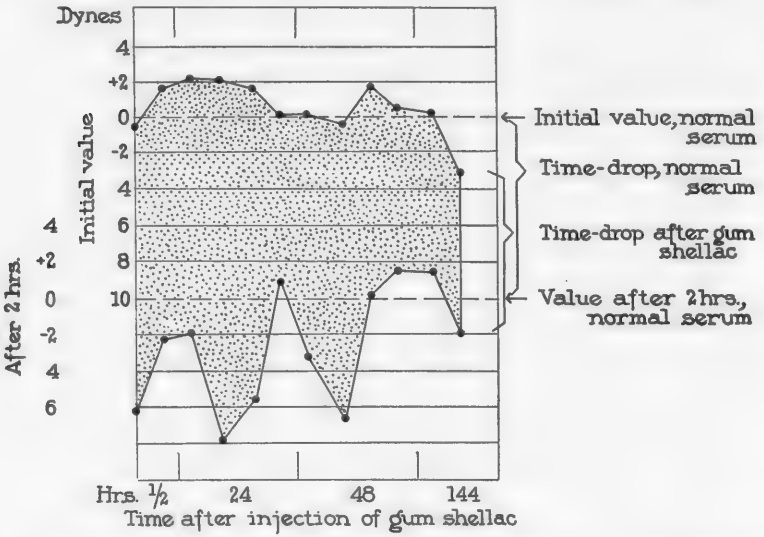
TABLE II.

Surface Tension of Dilutions of Rabbit Serum after Repeated Bleeding.

Rabbit No.	Dilution of serum	First bleeding			Subsequent bleedings after											
					½ hour			24 hours			48 hours			144 hours		
		Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop	Initial value	After 2 hrs.	Time-drop
		dynes	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*	*dynes*
8	1: 5,000	74.6	66.5	8.1	73.3	64.1	9.2									
	1:10,000	75.4	68.5	6.9	75.1	65.4	9.7									
	1:15,000	75.5	70.3	5.2	75.3	66.2	9.1									
10	1: 5,000	71.4	63.6	7.8	74.0	63.2	10.8				73.7	63.9	9.8			
	1:10,000	73.0	65.7	7.3	75.4	65.6	9.8				75.2	67.9	7.3			
	1:15,000	74.1	67.1	7.0	75.7	66.3	8.4				75.6	68.9	6.7			
4	1: 5,000	74.3	63.9	10.4				73.3	63.7	9.6	70.9	60.7	10.2	75.6	66.8	8.8
	1:10,000	76.3	66.5	9.8				76.2	66.5	9.7	71.5	63.2	8.3	76.5	68.8	7.7
	1:15,000	76.4	68.2	8.2				76.6	68.7	7.9	71.6	65.1	6.5	76.7	71.4	5.3
6	1: 5,000	75.6	66.8	8.8							71.6	63.5	8.1	71.4	63.6	7.8
	1:10,000	76.5	68.8	7.7							74.4	65.4	9.0	73.0	65.7	7.3
	1:15,000	76.7	71.4	5.3							74.1	64.8	9.3	74.1	67.1	7.0
13	1: 5,000	68.2	61.0	7.2				73.9	64.4	9.5						
	1:10,000	68.8	60.8	8.0				75.4	67.1	8.3						
	1:15,000	71.5	65.2	6.3				75.5	68.5	7.0						
14	1: 5,000	74.3	63.3	11.0				71.7	62.6	9.1						
	1:10,000	75.2	66.4	8.8				72.5	63.6	8.9						
	1:15,000	75.5	67.8	7.7				74.6	64.6	10.0						

EXPERIMENTAL DATA.

The estimations of the surface tension of diluted serum before and after the injection of gum shellac solution in eight rabbits are presented in Table I. The figures given are the average of two measurements at 1:5,000 and 1:15,000 dilutions and of three at 1:10,000. In two animals blood was drawn ½ hour after injection of gum; in four animals, 24 hours; in four, 48 hours; and in two, 144 hours after injection of gum.



TEXT-FIG. 1. The stippled area represents the time-drop of 1:10,000 dilution of serum after the injection of gum shellac while the area between the two parallel lines corresponds to the time-drop of normal serum.

There was a distinct increase in the value of the time-drop ½ hour after injection in both animals; an increase in three of four 24 hours after injection; and in two, 48 hours after injection. In both animals bled after 6 days, the time-drop had returned to normal.

The data of six control experiments are presented in Table II. In order to determine whether the injection of any colloid was followed by change in the surface tension of serum, one animal was given 5 cc. of 6 per cent gum acacia solution and one, 5 cc. of diphtheria

antitoxin intravenously after the first bleeding. No change in surface tension of the serum was detected.

TABLE III.

Change in Time-Drop of 1:10,000 Dilution of Serum after Intravenous Injection of Gum Shellac Solution.

Rabbit No.	Before injection	After injection of 5 cc. of gum shellac solution			
		½ hour	24 hours	48 hours	144 hours
	dynes	*dynes*	*dynes*	*dynes*	*dynes*
7	7.9	13.7			
9	8.3	12.2		9.4	
12	6.6		10.7		
3	10.5		9.5	14.0	9.0
5	9.0			15.1	8.3
11	9.7			8.8	
15	5.7		15.4		
16	8.8		15.8		
Average.....	8.3	12.9	12.8	11.8	8.6

Change in Time-Drop of 1:10,000 Dilution of Serum with Repeated Bleedings and Injection of Other Colloids.

Rabbit No.	First bleeding	Subsequent bleedings after			
		½ hour	24 hours	48 hours	144 hours
	dynes	*dynes*	*dynes*	*dynes*	*dynes*
8	6.9	9.7			
10	7.3	9.8		7.3	
4	9.8		9.7	8.3	7.7
6	7.7			9.0	7.3
13	8.0*		8.3		
14	8.8**		8.9		
Average.....	8.0	9.7	8.9	8.2	7.5

* 5 cc. of 5 per cent acacia intravenously immediately after bleeding.

** 5 cc. of diphtheria antitoxin immediately after bleeding.

Inspection of these tables shows that the maximum time-drop was recorded at 1:5,000 more frequently than at 1:10,000. This we believe to be due to technical errors. The curvature of the watch-

glasses, although approximately uniform, was not calibrated, so that a monomolecular layer at the surface may not have been possible at 1:10,000 in some instances.

The changes in time-drop at 1:10,000 dilution in both control animals and after the injection of gum shellac solution are summarized

TABLE IV.

Rise of Surface Tension in Function of Time after Drop Due to Addition of 0.5 cc. Gum Shellac Solution.

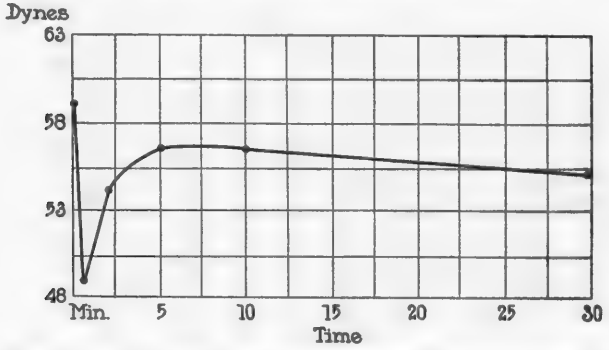
Time	Surface tension			
	Concentration of serum			
	Pure	1:10	1:20	1:100
	dynes	*dynes*	*dynes*	*dynes*
Before adding gum.....	59.0	61.7	60.7	60.8
After " "	49.6	50.8	50.8	50.6
" 2 min.....	54.0	55.1	53.5	51.9
" 5 "	56.8	57.0	56.7	54.4(?)
" 10 "	56.6	56.3	57.7	
" 30 "	55.3	55.0	57.0	51.6

Rise of Surface Tension in Function of Time after Drop Due to Addition of 0.025 cc. Gum Shellac Solution.

Time	Surface tension		
	Concentration of serum		
	Pure	1:10	1:20
	dynes	*dynes*	*dynes*
Before adding gum.....	60.8	60.3	60.1
After " "	53.6	53.5	50.3
" 2 min.....	56.0	57.7	55.0
" 5 "	56.4		56.2
" 30 "	56.4	58.0	58.4

in Table III and Text-fig. 1. In this figure the stippled area represents the time-drop of 1:10,000 dilution of serum after injection of gum, while the area between the two parallel lines corresponds to the time-drop of normal serum. In constructing the figure (3), the initial value of serum before injection of gum was taken as 0, thus representing the axis of abscissæ, and difference between the initial values

of surface tension of serum of the same animal before and after injection of gum shellac solution were plotted with respect to this axis. The same procedure was followed in plotting final values. When these two sets of figures are plotted on one graph, however arbitrary the distance may be between the two parallel abscissæ representing the initial value of normal serum and the value after 2 hours, the ratio between this area and that included between the curves representing initial and final values of serum after injection of gum, expresses the ratio between the values of the time-drop in both cases.



TEXT-FIG. 2. Rise of surface tension of pure serum after drop due to addition of 0.05 cc. of gum shellac solution.

Recovery after Lowering of Surface Tension by Gum Shellac Solution.

Du Noüy (5) has shown that after the addition of minute amounts of sodium oleate to serum there is a rapid recovery of surface tension due to the adsorption of the oleate by the serum molecules or micellæ. After the addition of 0.05 cc. of gum shellac solution to 2 cc. of pure or diluted serum there was rapid fall of about 10 dynes in surface tension and prompt recovery in dilutions less than 1:100 (Table IV and Text-fig. 2). The initial fall is only about half that produced by minute amounts of sodium oleate. The addition of 0.025 cc. of gum shellac solution produced a distinct, but less marked fall in the surface tension. The surface tension of the gum shellac solution itself was 46.8 dynes. The addition of 0.05 cc. of this solution to 2 cc. of 0.9 per cent NaCl lowered the surface tension from 76.2 to 50.1 dynes: 1 hour later it was 49.9 dynes.

This experiment shows that the gum acts like a surface-active substance. Since the du Noüy phenomenon of recovery is observed, the gum molecules must be adsorbed by the serum molecules.

A crude attempt was made to detect adsorption of surface-active substances from the gum shellac solution on red blood cells by their rate of sedimentation. No differences could be detected between the rate in blood to which 3 per cent of gum shellac solution was added, or in blood drawn 30 minutes after intravenous injection of gum shellac solution, and in the controls.

CONCLUSIONS.

Following the intravenous injection of a gum shellac solution which alters the peripheral blood picture there is an increase in the time-drop of the diluted serum. This is believed to indicate changes in the physicochemical state of the plasma.

This gum shellac solution behaves as a surface-active substance; its effect on the surface tension of serum is hindered by the du Noüy phenomenon of adsorption on serum molecules.

BIBLIOGRAPHY.

1. Muller, G. L., *J. Exp. Med.*, 1927, xlv, 753.
2. du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707.
3. du Noüy, P. L., *J. Exp. Med.*, 1923, xxxviii, 87.
4. du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.
5. du Noüy, P. L., *J. Exp. Med.*, 1922, xxxvi, 115.

ETIOLOGY OF OROYA FEVER.

VIII. Experiments on Cross-Immunity between Oroya Fever and Verruga Peruana.

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(Received for publication, December 30, 1926.)

A common etiologic origin of Oroya fever and verruga peruana was first indicated experimentally by the production of verrucous lesions in monkeys (*Macacus rhesus*) by inoculation of cultures of *Bartonella bacilliformis* isolated from the blood in a fatal case of Oroya fever.[1] Subsequent experiments with the same strain[2,3] gave evidence that the type of disease induced by the parasite was dependent upon two factors, the virulence of the strain, and the degree of susceptibility of the individual animal, various grades of infection being induced in the experimental animals, from a moderate or extensive local skin affection to a severe systemic disease terminating in fatal anemia. More recently,[4] there has been isolated from the skin lesion of verruga peruana a microorganism morphologically and serologically identical with *Bartonella bacilliformis* and having similar pathogenic properties. It remained to be proven that animals which had recovered from infection with the Oroya strain of *Bartonella bacilliformis* would be able to resist infection with the verruga strain.

The disease induced by *Bartonella bacilliformis* in *M. rhesus* is chronic in nature, but complete recovery may take place and is evident from (1) the subsidence of the febrile reaction, (2) the disappearance of the skin lesions, (3) the sterility of the blood and lymphatic glands, as shown by cultivation experiments, and finally (4) immunity to the homologous strain. All of the ten monkeys

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.
[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.
[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.
[4] Noguchi H., *J. Exp. Med.*, 1927, xlv, 175.

(nine *Macacus rhesus* and one chimpanzee) selected for the present investigation had been shown by cultivation of blood and lymphatic gland tissue to be free from organisms, and five of them had been proved immune to the homologous (Oroya) strain by one or more previous immunity tests. The final test of immunity against both strains was made with suspensions of nodular tissue from *M. rhesus* 41 (verruga strain, second passage) and *M. rhesus* 36 (Oroya strain, fifth passage), the Oroya strain being inoculated on the right eyebrow and abdominal skin, intradermally and by scarification, the verruga strain similarly on the left side. Both suspensions induced well developed lesions in a control monkey, the verruga strain being decidedly the more virulent locally, as shown in the protocol of this animal. In the ten animals which had previously passed through a course of infection with the Oroya strain no signs of infection could be induced.

M. rhesus 1 had been inoculated on Nov. 16, 1925, intradermally and intravenously, with the first generation cultures of the blood of Case S. A. 15.[1] *Bartonella bacilliformis* was cultivated from the blood several times during the course of disease, and nodules arose at the site of intradermal inoculation. The lesions had disappeared within 2 months, and on Feb. 22, 1926, cultures of blood and lymphatic gland tissue were negative. Mar. 9, 1926, the animal received intravenously and intradermally a mixture of cultures and a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 31, and Apr. 13 proved sterile. On Apr. 29 the animal was inoculated again, intradermally and by scarification, with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] On May 19, minute lesions appeared to be developing on the eyebrows, but the slight indurations disappeared, and blood and lymphatic gland tissue taken on June 1 yielded no cultures. The final test with both Oroya and verruga strains was made on June 3, the inoculation being made intradermally and by scarification. No lesions developed.

M. rhesus 2 was first inoculated Dec. 8, 1925, and had passed through a protracted course of infection with the Oroya strain.[1] The blood had yielded cultures on several occasions, and the nodules at the sites of intradermal injection on the eyebrows were still present on Mar. 27. They had disappeared by Apr. 27, and the blood and lymphatic gland tissue taken on that date were negative by culture test. On Apr. 29 the animal was reinoculated with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] No signs of local or systemic infection appeared, and blood and lymphatic gland tissue proved sterile on June 1. On June 3 the animal was inoculated intradermally and by scarification with both the Oroya and the verruga strains. Blood and lymph node cultures made June 22 and July 13 proved sterile. No local lesions developed.

M. rhesus 3 had been inoculated with the same material as *M. rhesus* 2 and had passed through a similar course of disease. Only traces of the local lesions were visible on Mar. 8, 1926. On Mar. 9 the animal was inoculated intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 22 yielded cultures of *Bartonella bacilliformis*, and small but definite lesions developed at the sites of local inoculation. These had disappeared by Apr. 28, however, and the blood was sterile. On Apr. 29 a suspension of nodular tissue from *M. rhesus* 25[2] and cultures were introduced intradermally and by scarification. No local reactions resulted, and on May 19 and June 1 blood and lymphatic gland tissue were sterile. On June 3 the animal received the inoculation of both the Oroya and the verruga strains, intradermally and by scarification. The blood remained sterile, and no local lesions developed.

M. rhesus 12[5] had been inoculated on Jan. 19, 1926, intravenously, subcutaneously, and by scarification with cultures derived from Monkeys 4, 6, and 7. The blood was positive by culture on two occasions, and there was high fever for 1 week, but the local lesions were never large, and they disappeared within a month. On Mar. 25 blood and lymphatic gland tissue were sterile. On Apr. 22 the animal was inoculated with a suspension of nodular tissue from *M. rhesus* 18,[2] cultures derived from Monkeys 7 and 20, and citrated blood of *M. rhesus* 25.[2] The blood was negative by culture on May 27, and lymphatic gland tissue was negative on June 1. No lesions developed. On June 3 the final test with both the Oroya and the verruga strains was made. The blood and lymphatic tissue were sterile when tested on June 22, and no lesions developed.

M. rhesus 16 had received two injections (Feb. 10 and Feb. 15, 1926) of killed cultures of *Bartonella bacilliformis*. No reaction resulted. On Mar. 6 active suspensions of the skin lesions from *M. rhesus* 5[2] and *M. rhesus* 18 were injected intradermally and by scarification. By Apr. 5 a large nodule had developed on the abdomen. On Apr. 14 it was larger and deep cherry red. By Apr. 28 the lesion had practically healed. On Apr. 29 the animal received cultures and a suspension of nodular tissue of *M. rhesus* 25[2] intradermally. Small papules appeared at the sites of injection on May 22, but they disappeared, and on June 1 no lesions were apparent. Blood and lymphatic gland tissue taken on this date were sterile. The final test with both the Oroya and the verruga strains was made on June 3. No lesions developed. The blood was sterile when tested on June 21.

M. rhesus 18[2] had been inoculated on Feb. 15, 1926, intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 5. Extraordinarily extensive lesions had developed by Mar. 17, and blood taken on Mar. 18 yielded cultures of *Bartonella bacilliformis* in dilutions as high as 1:100,000. The lesions persisted for many weeks but had become very small, pale, and fibrous by the middle of June. Blood and lymphatic gland tissue taken on May 15 yielded no cultures. No reaction followed the inoculation of the Oroya and verruga strains on June 3, and on June 22 the blood and lymphatic tissue were sterile by culture test.

M. rhesus 22, inoculated Mar. 5, 1926, intradermally and by scarification on

[5] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

eyebrows and abdomen with nodular tissue of *M. rhesus* 5. Apr. 26, blood diluted 1:1,000 yielded cultures of *Bartonella bacilliformis*. A nodule had developed on the right eyebrow, and by May 6 it was very large and pedunculated. The lesion had disappeared by June 1, and blood and lymphatic gland tissue taken on that date were sterile by culture test. The final test with the Oroya and verruga strains was made on June 3. No lesions developed during 4 weeks of observation, and the blood was sterile by culture test on June 22.

M. rhesus 26[5] had been infected by the bites of four ticks previously fed on *M. rhesus* 18, the ticks having been transferred from Monkey 18 to Monkey 26 on Mar. 29, 1926. Lymphatic gland tissue (inguinal) taken on Apr. 14 yielded cultures of *Bartonella bacilliformis*, as did also blood taken on Apr. 24. On May 28 blood and lymphatic tissue were both sterile. The animal was inoculated on June 3 with both the Oroya and the verruga strains. No lesions developed during 6 weeks of observation. The blood was negative by culture test on June 22.

M. rhesus 31 was inoculated intradermally Apr. 5, 1926, with a suspension of testicular tissue of Rabbit 1744[6] and nodular tissue of Dog 1.[6] Blood taken on Apr. 28 yielded cultures in a dilution as high as 1:10,000. The nodules were well developed at this time. May 22, the nodules were reduced in size. May 28, blood culture sterile. June 1, lesions had disappeared, blood and lymphatic tissue failed to yield cultures. June 3, final test with Oroya and verruga strains. No reaction. Blood sterile on June 22.

Chimpanzee (*Pan leucoprymnus*), had been inoculated Jan. 29, 1926, intradermally, subcutaneously, and by scarification, with mixture of cultures and suspension of nodule of *M. rhesus* 3. Blood positive in dilution of 1:10,000 Feb. 23, 1:100 Mar. 12. Large nodules developed on eyebrows and abdomen. The animal had recovered completely by May 1, and neither blood nor lymphatic gland yielded cultures of *Bartonella bacilliformis*. On June 3 the animal was tested for immunity against the Oroya and verruga strains in the same way as the nine *rhesus* monkeys. No lesions developed, and blood culture was negative on June 28.

The following protocol illustrates the effects produced in a non-immune control by inoculation of the materials used on June 3 for the final test against the Oroya and verruga strains of *Bartonella bacilliformis*.

M. rhesus 45 was inoculated June 3, 1926, with the same materials and in the same manner as the recovered monkeys, the Oroya strain being inoculated on the right eyebrow and abdominal skin, the verruga strain at corresponding sites on the left side. Several minute nodules were recognizable at the sites of inoculation on

[6] Both the testicular tissue of the rabbit and the nodule of the dog yielded cultures of *Bartonella bacilliformis;* the report of these and other experiments will be published later.

the left side within 10 days and had attained considerable size (8 × 20 mm.) 32 days after the inoculation. The reactions at the sites of inoculation with the Oroya strain did not become noticeable until 3 weeks after inoculation. The nodules reached their maximum size (4 × 8 mm.) by the middle of July, never attaining the extent of the lesions on the left side. All the lesions were similar in character, however, being of the usual subcutaneous, *mular* type. Cultures made with blood withdrawn on June 28, 1926, 25 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. All the lesions had healed by the middle of August.

The experiments demonstrate that *rhesus* monkeys which have recovered from infection with the Oroya strain of *Bartonella bacilliformis* are completely immune to the verruga strain. The results warrant the conclusion that the strains from Oroya fever and verruga peruana are identical, and that both conditions are manifestations of infection with *Bartonella bacilliformis*.

The duration of the infection in the present series of animals varied from 2 to 5 months. The cultural test of lymphatic gland tissue appears to be extremely important in determining whether or not the animal is free from infection, as has already been shown in another series of experiments,[7] in which it was found that two animals of a group of four which had apparently recovered still harbored *Bartonella bacilliformis* in certain organs 48 and 58 days, respectively, after inoculation. In this earlier series of animals convalescence was established as early as 30 days in some instances, while in others the infection was still active 68 days after inoculation. The duration of a fatal infection varies from 25 to 57 days.[7]

SUMMARY.

Nine monkeys (*Macacus rhesus*) and a chimpanzee which had recently recovered from an infection with the Oroya strain of *Bartonella bacilliformis* were tested for immunity against the verruga strain of *Bartonella bacilliformis* as well as against the homologous strain. Complete immunity to both strains was demonstrated. The result establishes the identity of the strains and is in agreement with the result of comparative serological study.

[7] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

The criteria of recovery include not only the subsidence of febrile reactions and local lesions but also a negative result of cultural tests of blood and lymphatic gland tissue.

Recovery may occur as early as 1 month after inoculation, but in most instances a period of 2 to 5 months is required for the completion of convalescence.

A STUDY OF PNEUMOCOCCI ISOLATED FROM HORSES UNDERGOING PNEUMOCOCCUS IMMUNIZATION.

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(Received for publication, January 10, 1927.)

In previous studies (1), the significance of tissue susceptibility to the pneumococcus was demonstrated experimentally in the development of the lesions of lobar pneumonia not only in very susceptible animals, but even in the highly immunized horse where extensive lesions of ulcerative infectious endocarditis were recorded. Many observations on the changes that occur in the pneumococcus growing in the presence of immune serum *in vitro* are found in the literature, and Rosenow (2, 3) early described the attenuation of pneumococci isolated from the lesions of human endocarditis. Endocardial lesions in horses undergoing immunization offer exceptional opportunities for this study.

In 1916, Stryker (4) studied the growth of pneumococci in their homologous immune sera, recording variation in agglutination, diminution in virulence, inhibition of capsule formation, increased susceptibility to phagocytosis, and changes in the absorptive power and antigenic properties of such cultures. Passage through susceptible animals restored these cultures to normal type. Griffith (5) also records the fact that virulent pneumococci are attenuated by growth in immune serum and that their immunological character is changed. He recognized alterations in colony development by distinguishing the virulent colony as "smooth" and the attenuated as "rough." Blake and Trask (6), also Reimann (7), have described similar changes in the pneumococcus grown in the presence of immune serum. Neufeld and Händel (8) observed that pneumococci were attenuated by cultivation at a temperature above 39°C. and by surface growth on unfavorable medium. More recently Yoshioka (9) has reported that pneumococci kept under conditions that lead to loss of virulence, such

as cultivation at 39°C., surface growth on unfavorable medium, and desiccation, suffer changes in morphology, cultural characteristics, capsule formation, lysis in bile, and especially in type specificity (agglutination).

It is the purpose of this paper to record observations made in the study of the pneumococci isolated from the blood stream in the course of pneumococcus immunization, or at autopsy on immunized animals which had died as a result of the development of pneumococcus processes in the heart or other organs. Such observations, presenting, as they do, definite evidence that there is an attenuation of the pneumococcus in the course of prolonged infectious processes in the tissues, are of special interest because pneumococcus infection, however serious and extensive it may be, is usually self-limited and terminates by crisis or rapid lysis and a complete resolution of the tissues. The opposite course, delayed resolution, prolonged balanced infection, and the persistence of the pneumococci, even in pure culture, for a long time, is occasionally found, although, in these conditions, secondary infections frequently develop.

Of all the pathogenic species, the pneumococcus is one of the most susceptible to changes in its environment. Examples of its sensitive reaction to environmental change are the rapidity with which its virulence is exalted by animal passage and, especially, the fluctuation in virulence which occurs in the course of 24 hours' growth in broth, a fluctuation directly related, apparently, to its vegetative activity (10). Although the alteration in the pneumococcus grown in the presence of immune serum, as recorded in the literature, appears to be due specifically to the action of the serum, it is difficult to determine to what extent it is effected by some specific interaction between the organism and its immune serum, and how much is brought about by the biologic changes which take place in a less favorable environment. Some of these changes are, possibly, of a specific nature, while others must be regarded as general in character.

Source of Cultures.

The cultures which are the subject of this study were isolated directly from horses undergoing immunization or from horses at

autopsy. The following descriptive notes concerning them have been abstracted from the laboratory records.

Strain 131.—Horse 131 was immunized by intravenous injection from September 26 to December 8, 1923, with a Type I culture, HL, obtained originally from Dr. G. W. McCoy of the Hygienic Laboratory, Washington, D. C. Following the last injection of 175 cc., the temperature of the animal was 104.4°F.; and 5 days later, 102.2°F. From a blood culture taken December 22, a Gram-positive, bile-soluble diplococcus which agglutinated in all three type sera was isolated. Later, another culture was obtained which gave similar reactions. January 6, 1924, the animal died. At autopsy, the lungs were found to be congested, with small areas of adhesion. In the heart, there was a marked thickening of one cusp of the mitral valve, ulceration and degeneration of the cusps of the aortic valve, with marked stenosis.

Strain 143.—Horse 143 was immunized with the same strain, I HL, from August 28, 1923, to July 17, 1924, when it was allowed to rest. Immunization was resumed October 29, 1924, with another and more virulent Type I strain, the standard Neufeld Culture 5. On December 24, 1924, 5 days after the last injection, the temperature was 101°F. and gradually rose to 104.6° on January 20, 1925. On January 2, a blood culture was taken from which was isolated a Gram-positive, bile-soluble diplococcus which grew like the pneumococcus in broth and fermented inulin, and which agglutinated non-specifically in all three type sera, as did the organism isolated from Horse 131. Other organisms which gave similar reactions were isolated from blood cultures 6 and 11 days later, but the growth of the last culture in broth was flocculent. The serum obtained from a bleeding of January 3 possessed a high protective titer when standardized for therapeutic use. January 14, the right hock was swollen and, on the 19th, valvular murmurs were noted. On the 20th, the temperature of the animal was 104.6°F., and, from a blood culture, another organism was obtained which was quite similar to the ones previously isolated and was also flocculent in its growth in broth. January 24, the animal died with ulcerative endocardial lesions. One cusp of the bicuspid valve was thickened; the other was thickened and covered with thick fibrinous exudate. The aortic valves showed marked ulceration and were covered with fibrinous exudate.

Strain 177-A.—The immunization of Horse 177 with the standard Type I No. 5 strain began July 7, 1924. November 4, the horse developed lameness. Its temperature ranged from 100–104.4°F. until it was bled out, January 30, 1925. January 24, a Gram-positive diplococcus with flocculent growth in broth was isolated from a blood culture. The agglutinative reaction of this organism was similar in character to that of the pneumococci isolated from the other horses. A mouse was inoculated with this culture, and the organism obtained at autopsy was a typical Type I pneumococcus in its reactions, and is designated as Strain 177-R hereafter. The possibility of intercurrent infection seemed unlikely, for

similar results were obtained with an organism taken from a blood culture, January 26. Similar organisms were obtained from blood cultures the 28th, 29th, and 30th.

Strain 177-R.—A mouse inoculated with 1 cc. of Strain 177-A, isolated January 23, 1925, died in 45 hours. The organism recovered at autopsy reacted like a typical Type I pneumococcus and was fatal for mice in 0.00001 cc. amounts. A typical Type I pneumococcus which killed mice in 0.000001 cc. doses, was obtained also from a mouse which received 1 cc. of the organisms isolated from Horse 177, January 26.

Strain 189.—Horse 189 was immunized with the standard Type I No. 5 strain, the first injection being given December 11, 1924, and the last July 31, 1925. From August 7 to August 12, the temperature of the animal ranged from 101–102.2°F. Blood cultures were sterile August 25, September 2 and 3. September 11 and September 16, a bile-soluble organism was isolated from blood cultures. It developed flocculent growth in broth and agglutinated in the three type sera, non-specifically. It did not kill a mouse in doses of 1 cc. The horse died September 17. At autopsy, the lungs were congested and hypostatic. The valves of the right heart were smooth and apparently normal but dark red in color. The bicuspid valves of the left heart were markedly thickened, and closure was incomplete. A white thrombus was attached to the auricular surface of the valve and extended through the auricle.

Strain 190.[1]—Horse 190 corresponded closely to Horse 189 as it also was immunized with the standard Type I No. 5 strain and was given first and last injections on December 11, 1924, and July 31, 1925, respectively. From August 7 to September 7, 1925, when the animal died, its temperature ranged from 101–102.6°F. Bile-soluble, Gram-positive organisms, which agglutinated in all three type sera, were isolated from blood cultures August 18 and September 3. 1 cc. failed to kill a mouse. At autopsy, an organism which gave similar reactions was isolated from the heart. The autopsy disclosed pneumonia which involved the apex of each lung and was more extensive in the right lung. There were no signs of pericarditis, but the endocarditis was marked, with extensive vegetation and destruction of the aortic and bicuspid valves.

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[1] The strains of pneumococci here recorded were also independently studied by Miss Helen R. Odell as to their virulence, type specificity, morphological and cultural reactions in the course of the examinations that are made as a part of the routine laboratory procedure. Although these tests preceded those described in this paper, the results were practically identical with the one exception that no capsules were demonstrated. Since these experiments were finished, two other atypical strains have been isolated from horses undergoing pneumococcus immunization. Both are Gram-positive, bile-soluble diplococci which agglutinate non-specifically in all three type sera. One strain, No. 150, quickly reverted to a typical Type I pneumococcus on mouse passage. The other strain, No. 201, did not kill mice in 1.0 cc. amounts.

These different strains were transferred promptly to serum semi-solid medium in which the biologic characteristics and virulence are most satisfactorily preserved for long periods of time. This medium consists of one part of 2.25 per cent agar added to five parts of serum, and has been used for this purpose since it was first described (11).

Morphology and Cultural Reactions.

All the cultures used in this study were Gram-positive cocci which grew in pairs or short chains with the typical morphology of the pneumococcus. The cultures which developed a flocculent growth in broth at the time of isolation soon formed the characteristic cloudiness of the pneumococcus when subcultured. The cultures on blood agar were all typical.

Capsules of these strains, with the exception of Strain 177-R, were demonstrated with difficulty. Those of Strain 131 were not demonstrable at all. Small capsules were shown on some organisms of the remaining strains at times, but were quite variable.

Inulin was fermented by all of the strains in subculture. The acidity of Nos. 131 and 189 exceeded that of the standard Type I culture. That of Nos. 143 and 177-R approximated the standard culture, whereas only weak reactions were obtained with Nos. 190 and 177-A.

Ox bile, one part to six parts of an 18 hour broth culture, dissolved all of these strains.

Sodium oleate 1:1000, one part to six parts of an 18 hour broth culture, dissolved Strains 131, 143, and 177-R in 30 minutes; Strains 177-A, 189, and 190 in 2 hours, at 35°C.

The vegetative activity, as compared with that of the standard Type I No. 5,[2] was studied in broth at 35°C. The usual lag (12) in the development of the freshly inoculated pneumococcus cultures was noted. This varied slightly in the different strains, but maximum growth developed with reasonable uniformity in from 6 to 7 hours. Following this, there was an interval of little or no change, but, in 24 hours, autolysis was apparent although varied in extent. This action was perhaps slightly more marked in the virulent cultures, the control

[2] Two strains of No. 5 were used for comparison; the one, a strain of exalted virulence maintained as a standard; the other, a strain which had become non-virulent as a result of repeated transfer in broth.

TABLE I.

Reactions of Atypical Pneumococci Isolated from Horses under Immunization with Pneumococcus Type I.

Strain	Source	Organism used in immunization	Capsule	Fermentation of inulin	Solubility in bile	Solubility in sodium oleate	Precipitation reaction: Type sera I	II	III	131	Normal horse	Agglutination reaction: Type sera I C	I M	II C	II M	III C	III M	131 C	131 M	Normal horse C	Normal horse M	Virulence: No. mice inoculated	Dose	No. mice died	Phagocytosis: Type sera I	II	III	131	Normal horse
																							cc.							
131	Horse 131	I HL No. 5D	−	++	+	+	+	+	+	+	−	−	1:3200	−	1:10	−	1:200	−	1:3200	−	1:40	2	1.5	1	+	+	+	+	+
																						2	1.0	0					
143	Horse 143	I HL No. 5D I No. 5	?	+	+	+	+	+	+	+	−	−	1:3200	−	1:10	−	1:400	1:40	1:1600	−	1:40	2	1.0	0	+	+	+	+	+
177-A	Horse 177	I No. 5	?	Tr.	+	+	+	+	+	+	−	−	1:3200	−	1:20	−	1:400	1:160	1:1600	1:2	1:40	2	1.0	2	+	+	+	+	+
																						2	0.5	1					
																						2	0.1	0					
177-R	Mouse inoculated with No. 177-A		+	+	+	+	+	−	−	−	−	1:20	1:160	−	−	−	−	−	−	−	−	2	0.000001	2	+	−	−	−	−

189	Horse 189	I No. 5	?	++	+	+	−	−	−	+	−	−	1:3200	−	1:20	−	1:400	−	1:3200	−	1:40	2 2	1.5 1.0	1 0	+	+	+	+	+					
190	Horse 190	I No. 5	?	Tr.	+	+	−	−	−	+	−	−	1:3200	−	1:20	−	1:200	−	1:3200	1:2	1:40	2 2	1.5 1.0	1 0	+	+	+	+	+					

Readings of precipitation and agglutination tests were made after 2 hours' incubation at 37°C.

The dilutions recorded are final dilutions. The highest dilutions used were 1:3200 for the type sera and 1:40 for the normal horse serum control.

C denotes the weakest dilution in which there is complete agglutination and M the highest dilution used in which agglutination can be detected by the unaided eye.

Small, undefined capsules were at times demonstrated on some organisms of Strains 143, 177-A, 189, and 190.

The precipitation reaction in undiluted serum developed slowly and irregularly in different tests.

The agglutination reaction also developed slowly and the clumps were small.

Strain 177-A, which was more virulent for mice than were Strains 131, 143, 189, and 190, quickly reverted to a normal Type I on mouse passage, one passage in most cases being sufficient for the change. No. 177-R, the form of No. 177-A recovered from a mouse, is typical in all its reactions.

No. 5, and No. 177-R, but the differences in degree were slight, and autolysis appeared earlier and was more marked in the cultures of Strain 143, which was not virulent for mice in 1 cc. doses.

Virulence.

The virulence of all the strains was tested by the inoculation of mice and rabbits. Rats were also used in testing Strain 131.

Mice survived inoculations of 1 cc. of Strains 131, 143, 189, and 190. Of two mice receiving 0.5 cc. of Strain 177-A, one died. From it was isolated an organism which proved to be a typical Type I pneumococcus in its reactions and which killed mice in doses of 0.000001 cc. within 48 hours.

Rats and rabbits survived inoculations of from 1 cc. to 5 cc. of Strain 131 given intravenously. Rabbits also survived intravenous inoculations of 5 cc. of Strains 143, 189, and 190 and of 3 cc. of Strain 177-A.

Immunological Reactions.

In Table I the immunological and other reactions are shown.

Precipitation reactions of the clear supernatant fluids after centrifugalization of 18 hour cultures of Strains 131, 143, 177-A, 177-R, 189, and 190, were tested with horse serum of Types I, II, and III, and with No. 131 rabbit serum.

The culture broth of Strain 177-R precipitated only in Type I serum. The precipitation reaction of the other strains in undiluted serum developed very slowly. In most cases, no reaction was apparent after 30 minutes' incubation. The precipitation was irregular and incomplete except with Serum 131 which generally gave a strong reaction after 2 hours' incubation. The reaction of Strains 131, 143, and 177-A in Type III serum was often as good as, and sometimes better than, in Type I serum. Culture broth of Strains 189 and 190 precipitated only in Strain 131 serum.

Agglutination reactions with salt solution suspensions of a density equal to barium sulfate standard No. 4 from 18 hour broth cultures, developed in all the above sera with all of these strains except No. 177-R which agglutinated only in Type I serum. In most cases, the clumps were small and agglutination slow in developing. The reaction

was seldom complete except in Strain 131 serum. Agglutination occurred in much lower dilution in Type II serum than in Type I or Type III serum. Normal horse serum showed agglutination through a 1:40 dilution.

Phagocytosis of Strains 131, 143, 177-A, 189, and 190 occurred in all type sera, No. 131 serum, and normal horse serum with leucocytes of the dog. Strain 177-R was phagocyted only with Type I serum.

Precipitation tests were made with three sera produced in rabbits against Strain 131 and with supernatant broth, after centrifugalization of 18 hour broth cultures of the strains Type I No. 5, Type I HL, Type II No. 5C, and Type III No. 5A, and of Strain 131. Normal serum was used as a control. At the end of 2 hours at 37°C., Strain 131 had reacted with all three sera. After standing overnight in the cold room, all types had precipitated in the Strain 131 sera.

Agglutination was tested with organisms of Types I, II, and III, and Strain 131, and the three sera produced against No. 131. Types I, II, and III organisms agglutinated slightly in one No. 131 serum diluted 1:10. Strain 131 agglutinated in the highest dilution used of all three sera, 1:3200, but it also agglutinated in normal horse serum, 1:40, the highest dilution used.

Phagocytosis of Type I No. 5 and HL No. 5D, Type II No. 5C, and Type III No. 5A did not take place in the presence of sera produced against Strain 131.

Protection against the standard Type I No. 5 strain and Type I HL was not obtained with a serum produced against Strain 131.

Soluble Specific Substances of Strain 131.

The extent of the attenuation of these pneumococci, together with their irregular serologic reactions in immune and in normal serum, suggested a study of their specific soluble substances in comparison with those of the standard Type I strain. This was undertaken by Dr. Anthony Kozlowski, but the work was interrupted by his return to Poland.

The observations of Heidelberger and Avery (13, 14) were confirmed in that the specific soluble substance described by them was obtained from the standard Type I Strain 5. From this attenuated Type I strain (No. 131) substances were obtained which reacted specifically

not only in Type I serum but also in Type III serum; also, substances which precipitated non-specifically in all three type sera and in serum produced against Strain 131.

Although quite large amounts of Strain 131 organisms were used, and numerous attempts made, it was not possible, with identical methods, to isolate a sufficient amount of substance, which would react with only one type serum and be entirely free from protein derivatives, to establish its chemical character. It is hoped that further studies will indicate more definitely the nature of the chemical changes in the specific substances which are associated with the attenuation of the culture. For the present it is important to note, however, that the production of the specific soluble substances was altered in the attenuated cultures.

SUMMARY.

It has been shown that the pneumococcus multiplying in the tissues of the immunized animal (horse) becomes attenuated: loses, in varying degrees, its virulence, capacity of capsule formation, susceptibility to phagocytosis, and type specificity. The antigenic activity as an immunizing agent and the production of "soluble specific substance" are also altered. In some instances, the typical pneumococcus characteristics may be quickly restored by one or two passages through a susceptible animal (mouse). In others, virulence is not recovered and the organism remains atypical.

Whether these changes are to be attributed to the specific action of immune bodies in the tissues, or are to be considered as the result of some biologic adaptive process to an adverse environment, has not been determined. Proof of the specific action of immune bodies is possibly open to question, whereas it is well known that virulence and with it some other characteristics are profoundly affected under unfavorable conditions in the absence of immune bodies, notably when the organism is grown at elevated temperatures or in certain unsuitable media.

These studies of pneumococci isolated from the infected immunized horse provide opportunities for further investigation of the significance of changes in virulence, type specificity, and formation of "soluble specific substances" in various forms of pneumococcus infection.

BIBLIOGRAPHY.

1. Wadsworth, A. B., Studies on pneumococcus infection in animals. Second paper: Action of immune sera on pneumococcus infection, *J. Exp. Med.*, 1912, xvi, 78; A study of the endocardial lesions developing during pneumococcus infection in horses, *J. Med. Research*, 1918–19, xxxix, 279.
2. Rosenow, E. C., Immunological and experimental studies on pneumococcus and staphylococcus endocarditis ("chronic septic endocarditis"), *J. Infect. Dis.*, 1909, vi, 245.
3. Rosenow, E. C., A study of pneumococci from cases of infectious endocarditis, *J. Infect. Dis.*, 1910, vii, 411.
4. Stryker, L. M., Variations in the pneumococcus induced by growth in immune serum, *J. Exp. Med.*, 1916, xxiv, 49.
5. Griffith, F., The influence of immune serum on the biological properties of pneumococci, *Rep. Pub. Health and Med. Subj., Ministry of Health, No. 18*, 1923, 1.
6. Blake, F. G., and Trask, J. D., Jr., Alterations in virulence and agglutination reactions of pneumococcus induced by growth in immune serum, *J. Med. Research*, 1923–24, xliv, 100.
7. Reimann, H. A., Variations in specificity and virulence of pneumococci during growth *in vitro*, *J. Exp. Med.*, 1925, xli, 587.
8. Neufeld, F., and Händel, L., Pneumokokken, in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 1912, iv, 527, 533.
9. Yoshioka, M., Untersuchungen über Pneumokokkenimmunität. II Mitteilung, *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 232.
10. Wadsworth, A. B., and Kirkbride, M. B., A study of the changes in virulence of the pneumococcus at different periods of growth and under different conditions of cultivation in media, *J. Exp. Med.*, 1918, xxviii, 791.
11. Wadsworth, A. B., A convenient method for growing and storing virulent pneumococci, *Proc. New York Path. Soc.*, 1903, iii, 113.
12. Chesney, A. M., The latent period in the growth of bacteria, *J. Exp. Med.*, 1916, xxiv, 387.
13. Heidelberger, M., and Avery, O. T., The soluble specific substance of pneumococcus, *J. Exp. Med.*, 1923, xxxviii, 73.
14. Heidelberger, M., and Avery, O. T., The soluble specific substance of pneumococcus. Second paper, *J. Exp. Med.*, 1924, xl, 301.

COMPARISON OF A VIRUS OBTAINED BY KOBAYASHI FROM CASES OF EPIDEMIC ENCEPHALITIS WITH THE VIRUS OF RABIES.

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(Received for publication, December 30, 1926.)

In 1924 there was a sudden outbreak of a form of epidemic encephalitis in Japan with a reported mortality rate of over 50 per cent which resulted in more than 4,000 deaths. During the epidemic Dr. Kobayashi[1] succeeded, after many attempts, in isolating from a single case of the disease a "virus" capable of indefinite transmission in experimental animals and very courteously sent samples preserved in glycerol to Dr. Flexner for study.

This virus is of peculiar interest. In the first place, because it is doubtful whether previous attempts to transmit the virus of epidemic encephalitis to animals have ever been successful.[2] Secondly, on account of the possibility that the virus of Japanese encephalitis may be of a different kind which, perhaps, may be more easily transmitted than the others, and be thus more readily studied.

The virus was isolated by Kobayashi through the subdural inoculation of rabbits with brain substance from a typical case of epidemic encephalitis diagnosed clinically as well as post mortem. The first symptoms were noted in rabbits on the third passage and subsequently in almost 100 per cent of rabbits inoculated subdurally or corneally. In his opinion, it resembles closely the virus of Koritschoner[3] and the virus isolated from dogs by Hoff and Silberstein.[4] He compared it with the fixed virus of rabies and found many and significant points of similarity as well as the following differences:

1. The encephalitis virus occurred, for the most part, in the brain and was not

[1] Kobayashi, R., *Japan Med. World*, 1925, v, 145.

[2] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[3] Koritschoner, R., *Virchows Arch. path. Anat.*, 1925, cclv, 172.

[4] Hoff, H., and Silberstein, F., *Z. ges. exp. Med.*, 1924–25, xliv, 257, 268.

demonstrated in the spinal cord or medulla; whereas that of rabies was found chiefly in the spinal cord.

2. When the encephalitis virus was inoculated on the cornea of 6 dogs, none developed the disease; but when 5 were inoculated subdurally, 4 were affected. With the virus of rabies, on the other hand, 2 out of 3 dogs developed characteristic symptoms after corneal inoculation and all after subdural inoculation.

3. The course of the disease produced by the encephalitis virus was prolonged, and that caused by the virus of rabies, acute.

Evidently it is important to extend the researches of Kobayashi to determine if possible the exact nature of his virus. The following immunological study was therefore made of the encephalitis virus which he sent us and of a fixed rabies virus obtained through the courtesy of Dr. Anna Williams of the New York City Department of Health.

EXPERIMENTS.

In order to test the cross-immunity, if any, existing between the encephalitis virus and the virus of rabies a procedure suggested by Dr. Williams' assistant, Mr. Tyler, was employed with slight modifications.[5]

The viruses were inoculated intracerebrally into a series of young rabbits. The brains of the animals which showed the most characteristic symptoms were used for the preparation of standard viruses of both kinds, which were preserved in ampules of non-alkaline glass. The M.L.D. were then ascertained by the intracerebral inoculation of guinea pigs weighing about 250 gm. These proved to be the same for both viruses, namely $\frac{1}{15,000}$ gm. of brain substance, although the incubation period was uniformly longer in the case of the encephalitis virus, which, on intramuscular injection, was much less potent than the rabies virus. For the first cross-immunity studies two series of 20 guinea pigs of approximately the same weight (250 gm.) were selected.

Series A was given a Pasteur treatment, consisting of 20 daily subcutaneous injections of inactivated fixed rabies virus. Inactivation was effected by making up an 8 per cent suspension of brain substance in 1 per cent carbolic acid in saline solution, and by incubating this at 37°C. for 24 hours. This was diluted with equal parts of sterile saline and, just before use, was diluted again 1 to 3. The amount injected was 0.5 cc. The "vaccine" was tested and found to be free from contaminating bacteria.

Series B received Pasteur treatment in the same way with the inactivated encephalitis virus.

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[5] Help received from Mr. Peter Haselbauer was invaluable.

The animals were left for 1 week after the conclusion of treatment before testing for cross-immunity. The injections were all made intracerebrally and the injected viruses were tested for bacterial contamination and found to be free from it. The symptoms of those succumbing were identical following the action of both viruses. After an incubation period of about 3 days, the temperature rose to about 105°F. on the 4th or 5th day. The animals then showed excessive salivation, tremors, convulsions and paralyses. The temperature commenced to fall on the 6th or 7th day and became rapidly subnormal—94° or 95°F.—when the animals died.

TABLE I.

The Action of Encephalitis Virus upon Animals after Pasteur Treatment with Rabies Vaccine.

Experiment Pasteur treatment with fixed rabies vaccine + encephalitis virus	*Control* Pasteur treatment with fixed rabies vaccine + fixed rabies virus	*Control* Untreated normal animals + encephalitis virus	*Control* Untreated normal animals + fixed rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
No characteristic symptoms or deaths	2 animals receiving 2 M.L.D. developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died

An examination of the results which are set forth in Table I shows:

1. That six animals which received Pasteur treatment with the rabies virus resisted subsequent inoculation with the encephalitis virus even to the amount of 3 M.L.D.

2. That the Pasteur treatment with the rabies virus produced a measure of immunity against the rabies virus itself.

3. That untreated normal animals generally succumb to the action of the encephalitis virus showing that the sample of encephalitis

TABLE II.

The Action of Fixed Rabies Virus upon Animals after Pasteur Treatment with Encephalitis Vaccine.

Experiment Pasteur treatment with encephalitis vaccine + fixed rabies virus	*Control* Pasteur treatment with encephalitis vaccine + encephalitis virus	*Control* Untreated normal animals + fixed rabies virus	*Control* Untreated normal animals + encephalitis virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
2 animals 4 M.L.D. virus	2 animals 4 M.L.D. virus	1 animal 4 M.L.D. virus	1 animal 4 M.L.D. virus
One 1 M.L.D. animal died of some cause unknown 11 days after injection	One 2 M.L.D. animal died from peritonitis resulting from intestinal perforation. The others were unaffected	One 1 M.L.D. animal was found dead, no characteristic symptoms having been noted	One 3 M.L.D. animal developed characteristic symptoms and died
One 2 M.L.D. animal developed characteristic symptoms and died			
One 3 M.L.D. animal developed characteristic symptoms and died			
Two 4 M.L.D. animals developed characteristic symptoms and died			

virus has retained its potency. The M.L.D. originally determined is too low because during the experiments, which extended over a period

of more than a month, the animals gained on the average 50 gm. in weight, in consequence of which 1 M.L.D. was not effective.

4. That untreated normal animals likewise succumb to the action of the fixed rabies virus indicating, in the same way, that it likewise has retained its potency. The fact that 1 M.L.D. was not effective is to be explained on the basis above mentioned of the increase in weight of the animals.

TABLE III.

The Action of Fixed Rabies Virus upon Animals 6 Days after Last Immunizing Dose with "A" and "B" Encephalitis Vaccines.

Experiment Pasteur treatment with encephalitis vaccine "A" + rabies virus	*Experiment* Pasteur treatment with encephalitis vaccine "B" + rabies virus	*Control* Untreated normal animals + rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus
One 3 M.L.D. animal died with typical symptoms*	All recovered but one had slight tremors	The animals receiving 1 and 3 M.L.D. died with typical symptoms.* The animal receiving 2 M.L.D. exhibited tremors, and became ataxic, but recovered

* Examined histologically.

It seems clear, therefore, that the action of the encephalitis virus was inhibited by Pasteur treatment with rabies vaccine so that a one-sided immunity exists.

The observations in Table II were designed to show whether the converse also holds. They are arranged in the same manner, but are less concise, because only 3 out of 8 animals, which received the Pasteur treatment with the encephalitis virus, resisted inoculation with the rabies virus. The controls are similar.

A further attempt was then made, along slightly different lines, to answer the same question as to whether it is possible to protect

animals against the rabies virus by Pasteur treatment with the encephalitis virus.

Instead of making one encephalitis "vaccine," as in the earlier experiments, two were made (designated "A" and "B") each from the brain of a typical case of the disease in a rabbit. The dose of vaccine was increased from 0.5 cc., used in the previous experiments,

TABLE IV.

The Action of Rabies Virus 9 Days after Pasteur Treatment with Encephalitis Vaccines "A" and "B."

Experiment Treated with vaccine "A" + rabies virus	*Experiment* Treated with vaccine "B" + rabies virus	*Control* Treated with vaccine "B" + encephalitis virus	*Control* Normal untreated + rabies virus	*Control* Normal untreated + encephalitis virus
2 animals received 1 M.L.D. virus	2 animals received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus
2 animals received 2 M.L.D. virus	2 animals received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus
2 animals received 3 M.L.D. virus	2 animals received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus
The 2 M.L.D. animal died with typical symptoms*	No characteristic symptoms: all recovered	No characteristic symptoms: all recovered	All died with typical symptoms*	The 1 M.L.D. animal developed tremors but recovered: the others died with typical symptoms*

* Examined histologically.

to 0.6 cc. The M.L.D. were so calculated as to apply to the animals after the period of immunization when each had gained about 50 gm. in weight. Both vaccines were employed in two sets of experiments and each set of animals thus treated was tested for immunity, respectively, 6 and 9 days after the last immunizing dose.

The results of the first set are detailed in Table III and it is evident that both encephalitis "vaccines" conferred a high degree of

immunity against the action of the rabies virus. The second set of experiments, recorded in Table IV, seems entirely to confirm this result. In other words, there is reason to believe that cross-immunity is demonstrable between the virus isolated by Kobayashi from a case of epidemic encephalitis and the fixed virus of rabies.

Although the immunological properties of the two viruses are thus apparently identical the action of the rabies virus is more rapid and on intramuscular injection more fatal. For example, 10 guinea pigs, which received intramuscularly at the back of the neck 0.5 cc. of a 10 per cent brain emulsion containing rabies virus, all developed characteristic symptoms and died. Whereas 10 other guinea pigs of about the same size which received similar doses of the encephalitis virus all lived. Differences of this kind may presumably be explained on the supposition that the two viruses, though of the same nature, possess different degrees of virulence.

Histological Studies.

The brains of 9 animals (marked with an asterisk in the tables) were examined after fixation in Zenker's fluid and coloration with Giemsa's stain. It was found that the lesions caused by the encephalitis virus and the rabies virus were identical.[6] Similarly 2 young dogs injected intracerebrally with the encephalitis virus developed symptoms indistinguishable from the familiar symptoms of

[6] Some of the rabbits employed exhibited lesions occasioned by another and wholly different disease, namely, spontaneous encephalitis. These lesions, however, do not constitute a source of error, once their existence is recognized, because they are of slow development and are sometimes associated with a specific and easily recognizable parasite.

In this connection it is desirable to supplement a foot-note in a recent paper on the geographic distribution of spontaneous encephalitis (Cowdry, E. V., *J. Exp. Med.*, 1926, xliii, 730). The note is as follows: "In addition, twenty-four Swedish rabbits, which had been sent to Dr. Flexner by Dr. Kling of Stockholm, were thoroughly examined. Two showed encephalitis and nephritis; one, encephalitis only, and another, only nephritis. No encephalitozoa were observed." It should be added that: "seventeen of these rabbits had been inoculated with brain substance sent by him to us, that the lesions mentioned were restricted to these inoculated animals and that the remaining seven rabbits were employed for control purposes and showed neither symptoms nor lesions."

furious rabies and died. In order to make sure of the comparison a third dog received an intracerebral injection of the street rabies virus and behaved in precisely the same way. Large and typical inclusion bodies were observed by a variety of methods in the brains of all 3 dogs.

CONCLUSION.

Since the symptoms produced in experimental animals by the encephalitic virus of Kobayashi and by the virus of rabies are similar, and are accompanied by lesions which are indistinguishable, and since a cross-immunity is demonstrable between the two viruses, the conclusion is advanced that the specimen of so called encephalitis virus isolated by Kobayashi is in reality a specimen of rabic virus.

THE OCCURRENCE OF DEGRADED PNEUMOCOCCI IN VIVO.

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(Received for publication, January 12, 1927.)

It has been well demonstrated that under certain experimental conditions *in vitro* pneumococci undergo variations. It is of great importance to determine whether variants ever appear *in vivo* and if so, under what conditions. This is especially important since several investigations have suggested that recovery from pneumococcus infection may depend largely upon a change of virulent pneumococci into avirulent and phagocytable forms. This paper describes attempts to determine the experimental conditions favoring a change of virulent (S) pneumococci into avirulent (R) forms within the animal body.

It has been repeatedly shown (1–3) that by subjecting type-specific (S) pneumococcus strains to various unfavorable cultural environments *in vitro*, certain biological characteristics of the bacteria are profoundly affected. The changed bacteria no longer elaborate the specific carbohydrate element upon which type specificity depends and they are therefore agglutinable in heterologous as well as homologous antipneumococcus serum (4), they are no longer virulent for mice, and they are easily phagocyted (5, 6). These organisms have been designated R pneumococci by Griffith on account of the rough surface of the colonies which they form when grown on solid media, in contradistinction to the smooth surfaced colonies formed by S or typical virulent pneumococci. This difference renders the two forms distinguishable microscopically and often macroscopically when colonies of both forms are present on the same blood agar plate.

An intermediate form which is somewhat less virulent than the original S strain and also possesses slight serological differences has also been described (7). By animal passage it is possible to restore the original virulence and type specificity of this form. On the other hand, by placing it under unfavorable cultural environment the intermediate

form is easily converted into the R form. It would seem that the intermediate organisms represent a transitional stage between the S and the R forms.

Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy. But in case the change from S to R does take place *in vivo* it seems probable that R forms would be phagocyted and destroyed as soon as they appear. Therefore, to determine the presence of the R forms *in vivo*, it would probably be necessary to employ a technic under which the infection would remain localized over a considerable period of time without killing the animal, and under which the free access of phagocytes to the bacteria would be inhibited. It was thought that these conditions might be obtained by embedding subcutaneously in the experimental animal an agar mass inoculated with a virulent strain of Pneumococcus.

Method.—15 cc. of melted nutrient agar was inoculated with 0.5 cc. of a young broth culture of Pneumococcus. A wire loop about 4 cm. in diameter was pressed firmly over a shaved area of skin of the anesthetized animal and the fluid agar injected subcutaneously into the area enclosed by the loop. Ice was applied until the agar had solidified into a firm hemispherical nodule. Observations were made from time to time by puncturing the focus with a hypodermic needle, aspirating some of the material, and plating it on blood agar plates. The plates were examined for S, R, and intermediate colonies after 12–18 hours incubation.

Experimental.

Agar Foci in Dogs.—Dogs were first employed since these animals have a natural relative immunity to Pneumococcus, and were likely to withstand infection over a considerable period of time. Two dogs were injected with agar containing Type I pneumococci according to the method described. Material was aspirated from the foci at intervals from the 1st to the 14th day after injection. But R forms never appeared on the culture plates made from the material at any time, although S forms were constantly present in large numbers. The dogs soon became ill and S pneumococci were recovered from the blood stream on several occasions. In a few days abscesses formed at the site of injection in both animals and by the 10th day the skin

over the foci ruptured and thick pus was discharged. About the 14th day the lesions began to heal, the pneumococci disappeared and the dogs recovered. After a rest period of 2 weeks the same dogs, which were now assumed to have an additional degree of immunity, although specific agglutinins were not demonstrable in the blood serum, were reinjected with infected agar. A repetition of the previous events occurred. The dogs recovered and no R forms were encountered either from the foci or from the blood stream. While it was impossible with this technic to demonstrate the appearance of R forms in dogs, it should be mentioned that 11 years ago Bull (8) discovered that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. In one dog which lived for 10 days before dying with meningitis, pneumococci isolated from the blood on the 9th day "grew in chains and were non-virulent."

Agar Foci in Rabbits.—The same technic was then employed with rabbits instead of dogs, and instead of using a Type I strain, a Type III strain was employed. This type was used because rabbits have a high degree of resistance to Type III pneumococci, in contrast to their susceptibility to Types I and II, and it was thought that animals infected with this type would live longer and the bacteria would therefore have a greater chance to undergo variations. The special strains employed have been shown to readily undergo variations *in vitro*. Three rabbits were used. Daily examination of the foci by aspirating and plating material from the foci on blood agar plates showed that only S forms were present. The number of pneumococci gradually diminished until no more were demonstrable by the 6th day after injection.

Agar Foci in Guinea Pigs.—In a similar manner six guinea pigs, actively immunized against Type I pneumococci, were injected with agar containing Type I pneumococci. Repeated cultures from the foci showed the presence of S forms alone until the 5th day after injection when eight R colonies were found on a blood agar plate seeded from the focus of one guinea pig. R colonies were subsequently recovered from the foci of the other five guinea pigs at intervals up to 6 weeks after injection. In all instances however, the S colonies greatly outnumbered the R forms and were recovered from the foci for 6 weeks, or until the agar was absorbed. Normal (unimmunized)

guinea pigs were also tested but died from pneumococcus septicemia too soon to be suitable for the experiment.

In spite of the mass of agar surrounding the pneumococci in the subcutaneous foci, the object of preventing the access of phagocytes was not attained. Stained films made from the aspirated agar showed the constant presence throughout the agar mass of many phagocytes containing pneumococci.

As mentioned above, it was assumed that the R forms, being easily phagocyted, were rapidly destroyed or removed from the site of infection. To test this assumption, agar masses inoculated with a heavy suspension of R pneumococci were injected subcutaneously into two guinea pigs. It was found that the R forms were recoverable for 5 weeks after injection or until the foci had been completely absorbed. Although microscopic examination revealed the presence of great numbers of phagocytes, they apparently had but little influence in removing the R forms as long as the focus remained.

In order to eliminate the activity of phagocytes entirely, the technic was then modified as follows: Agar, which was inoculated with S pneumococci, was enclosed in a glass vial sealed with a collodion membrane. The membrane was an effective barrier against phagocytes but permitted the diffusion of fluids.

Method.—5 cc. wide mouth vials were filled, nearly to the top, with melted agar and the mouths were covered with thin muslin caps. After sterilization the agar was inoculated with 0.1 cc. of pneumococcus broth culture by means of a long hypodermic needle, and the muslin-covered mouths were dipped in a thin solution of collodion. The vials were then immersed in 95 per cent alcohol for several minutes to render the membrane more permeable. The sealed vials were embedded subcutaneously in rabbits. Repeated observations were made by inserting a long hypodermic needle through the skin of the animal and the membrane of the vial, aspirating some of the agar, and plating it on blood agar.

Control vials were prepared in the same manner but, instead of being placed *in vivo*, they were immersed in normal rabbit serum in large test-tubes. The tubes were kept in the incubator at 39°C. which was considered to be the average temperature of the vials while embedded in the rabbits. The serum was changed weekly.

Vials containing agar inoculated with cultures derived from a single diplococcus of a Type I strain were inserted subcutaneously in eight normal and three passively immunized rabbits and allowed to remain

for several weeks. Eight vials similarly prepared were immersed in normal serum and kept in the incubator as controls. Agar was aspirated from the vials and plated on blood agar at intervals of from 1 to 5 days. Several of the normal (unimmunized) rabbits soon died from pneumococcus septicemia due to infection resulting from leakage of the vials after aspiration. The unimmunized rabbits which survived were observed over a period of 8 weeks. Their serum did not show the presence of agglutinins for Type I pneumococci even at the end of this time.

R colonies were invariably found on plates made from all of the vials embedded in rabbits. They appeared, often with the intermediate forms, as early as the 3rd day after inoculation and were recoverable up to 8 weeks. The variant colonies were usually outnumbered by the S forms and in no instance were the S forms entirely replaced by them. It was rather surprising to find that both R and intermediate colonies were derived from the agar vials in normal and immunized animals alike.

The eight control vials inoculated at the same time with the same culture of Type I pneumococci were kept in the incubator. R colonies never appeared on plates made from these vials although the S forms remained during the period of observation.

Experiment with Type III Pneumococcus.—Three agar vials were inoculated with a Type III pneumococcus culture obtained from a single diplococcus from the blood culture of a pneumonia patient, and embedded subcutaneously in three rabbits. Frequent plating of the agar at intervals during a period of 5 weeks never revealed the presence of R or intermediate forms although S forms remained viable throughout.

This culture proved to be a strain especially refractory to modification as was determined by subjecting it to treatment *in vitro* by methods which invariably caused the appearance of R forms in other strains. Frequent search during 40 transfers in broth containing 5 per cent Type III antipneumococcus serum, or during 11 transfers in broth containing optochin yeast (9), or during 53 transfers on blood agar plates, did not reveal the presence of any R or intermediate forms. The culture remained unchanged in virulence and type specificity. Takami (10) has also encountered resistant strains of this

nature. He found that, of 28 strains, 12 never gave rise to variant forms even after prolonged subculture on blood agar.

Spontaneous Appearance of R Forms in Vivo.—Aside from the experimental production of the variant forms it is of interest to report the spontaneous occurrence of R forms *in vivo*. Through the courtesy of Dr. Mary B. Kirkbride of the New York State Department of Health, it was learned that atypical pneumococci were occasionally recovered from the blood stream of horses during the process of immunization with live virulent cultures for the production of antipneumococcus serum. The atypical strains were recovered from 6 to 16 months after the horses were first inoculated with a Type I pneumococcus culture. During this time the horses had a low grade fever, anorexia, loss of weight, rapid irregular pulse, heart murmurs, swelling and stiffness of the joints. The animals finally died and autopsy revealed an endocarditis present in all. Atypical pneumococcus strains from four of these horses were obtained and examined in this laboratory. They proved to have many characteristics in common with the variant or R strains previously studied. Each of the four strains produced colonies which differed slightly morphologically from one another and were distinguishable on blood agar plates. It was previously noted that in a number of instances, R colonies differing from one another in appearance were also recovered from the agar vials embedded in rabbits. The four horse R strains together with four strains derived from R colonies from the rabbit vials were tested for variations in virulence and agglutinability. All strains failed to kill mice in doses of 1 cc. of a young broth culture, were bile-soluble, and were serologically alike. The morphological colony differences between the various strains persisted even after repeated plating. Repeated (six) animal passages of the R strains failed to restore either virulence or specificity.

DISCUSSION AND CONCLUSION.

It is conceivable that a change from the virulent, non-phagocytable S form of Pneumococcus to the avirulent phagocytable R form may take place in pneumococcus disease, but the experiments here reported do not settle the question whether or not this is an important factor in determining the outcome in natural infection. It has been shown

experimentally that the degradation from the S form to the R form actually does take place in cultures of Pneumococcus growing in agar subcutaneously embedded in guinea pigs, in agar enclosed in vials subcutaneously embedded in rabbits, and spontaneously in the blood stream of infected horses. However, it was not possible in any of the experiments here cited to demonstrate the complete change from S to R pneumococci before the bacteria disappeared from the body. When the intermediate or R forms did appear, they were always accompanied and usually exceeded in number by the S forms and all three forms disappeared together. S organisms may disappear entirely without evidence of first going through the intermediate and R stages. On the other hand, contrary to expectations, pure cultures of R forms remained viable in subcutaneous foci for weeks although apparently freely accessible to the action of phagocytes. It seems of some significance that the R forms appeared early in the vials (inoculated with S pneumococci) in immunized and normal rabbits alike, indicating that the presence of demonstrable specific immune bodies was not alone responsible for the variation of the bacteria.

Of some importance also is the fact that R forms were never derived from similarly prepared control cultures growing *in vitro* at the same temperature and immersed in normal serum, although the S forms remained viable and unaltered for 6 weeks. It is likely that variations of pneumococci do not occur readily when S cultures are exposed to normal serum *in vitro*, especially when growing in closed vials under a diminished oxygen supply, for it has previously been shown (2) that only slight variation occurs even after prolonged (240) transfers in heterologous serum broth in the test-tube. It is possible, therefore, that the variation which occurred among pneumococci growing in agar vials embedded in normal rabbits was actually provoked by unknown influences in the living tissue fluids.

Although R forms have been shown to occur *in vivo*, no positive evidence can be derived from these experiments to prove that recovery from pneumococcus infection depends upon the degradation of the virulent S forms of pneumococci to the avirulent R forms and the subsequent destruction of the latter by phagocytes.

BIBLIOGRAPHY.

1. Griffith, F., *Rep. Pub. Health and Med. Subj., Ministry of Health, No. 18*, 1923, 1.
2. Reimann, H. A., *J. Exp. Med.*, 1925, xli, 587.
3. Amoss, H. L., *J. Exp. Med.*, 1925, xli, 649.
4. Reimann, H. A., *J. Exp. Med.*, 1926, xliii, 107.
5. Friel, A. R., *Pub. South African Inst. Med. Research, No. 5*, 1915, Jan. 26.
6. Stryker, L. M., *J. Exp. Med.*, 1916, xxiv, 49.
7. Blake, F. G., and Trask, J. D., Jr., *Abstr. Bact.*, 1923, vii, 353.
8. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 7.
9. Reimann, H. A., *J. Exp. Med.*, 1927, xlv, 1.
10. Takami, T., *Tohoku J. Exp. Med.*, 1925, vi, 41.

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

III. Resistance to Chemicals.

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(Received for publication, December 21, 1926.)

Reference has already been made to the minuteness of size of the virus of foot-and-mouth disease and to its electropositive charge in the first two papers of this series.[1,2] We believe that these two factors serve to explain in part the unusual resistance of the virus to certain chemicals. So great is its tenacity in the presence of strong reagents that one might interpret this phenomenon as a sign of the inanimate character of the incitant. But as the sequel will show these chemical actions are illusory.

Resistance to Chemicals.

Although filter-passing viruses are generally resistant to certain chemicals, the active agent of foot-and-mouth disease is exceptionally so. In this remarkable resistance a parallel has been found thus far in the virus of mosaic disease.

For example, Abe[3] found that 70 to 75 per cent alcohol precipitates the virus of foot-and-mouth disease, along with the albumens of infective blood, which can then retain its activity for 2 to 3 days in a dried state. Stockman and Minett[4] reported that 25 to 50 per cent alcohol does not destroy it after at least 3 days and 10 per cent, after at least 20 days. With regard to chloroform and ether, these writers state that 10 drops of chloroform to 5 cc. of 1:100 virus do not inhibit activity after at least 27 days; and 10 drops of ether to 3 cc. of filtrate do not destroy

[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

[2] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 685.

[3] Abe, T., *Z. Infektionskrankh. Haustiere*, 1925, xxviii, 111.

[4] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

it after more than 10 days. In similar proportions of acetone the active agent survives for at least 4 days, and in 50 per cent glycerol, as is the case with many filter-passing viruses, indefinitely. These remarkable conditions have also been confirmed in general by Bedson and Maitland.[5]

The results of our experiments are in agreement with those of Abe and the British investigators. For we have found repeatedly that in 20, 40, 50, and 60 per cent alcohol the virus either in ground infected pad tissue or in aspirated lymph remained alive for at least 26 hours. In 5 experiments, the virus admixed with practically undiluted acetone maintained its viability for 20, but not for 30 minutes. Nor was it killed by supersaturation with ammonium sulfate—the active agent was viable in the coagulum for at least 2 hours.

In view of the fact that bacteria generally are destroyed very rapidly by these agents (staphylococci in 1 minute by 60 per cent alcohol), we believed that it was sufficiently important to undertake a comprehensive series of experiments which might indicate whether the resistance to chemicals was real or merely masked. We were aware that under the conditions employed by earlier investigators, a comparison between the resistance of the virus and that of bacteria, such as staphylococci, could not be made. In the case of the latter the reagents were added to a pure culture or a material which was presumed to contain nothing but bacteria and the diluent; in that of the virus the chemicals were added to tissues or the solid and fluid constituents, mainly protein, of an inflammatory exudate, of which the virus occupied only a small part. Furthermore the chemicals to which the active agent is resistant are all capable of coagulating proteins. That the virus is readily adsorbed to these coagula will be shown in the following protocol.

Protocol 1. Control.—Infected guinea pig pads 24 hours after injection were ground with sand and a quantity of 90 per cent alcohol was added to make the final dilution of the latter 60 per cent. A heavy coagulum resulted. After 2 hours centrifugation at 2500 R.P.M., the clear supernatant fluid and the sedimented material were injected intradermally in 3 guinea pigs, respectively, for each of the 2 portions. After 24 hours all 6 showed typical primary lesions and after 48 hours, secondary vesicles in the mouth and pads.

[5] Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

Test.—At the same time, the coagulum of similar alcoholized material, which was active for at least 26 hours, was allowed to settle for 1 hour. The topmost and lowest portions were injected into guinea pigs with the result that all 4 animals exhibited primary lesions 24 hours after inoculation, and secondary lesions 24 hours later. The supernatant fluid was filtered through Berkefeld candles. The filtrate was free from virus.

This experiment is typical of 7 others. In the remaining tests the contact with alcohol before filtration covered a period of from 2 to 3 hours and the virus employed consisted of infective blood, ground pad tissue, or aspirated lymph.

To summarize the results of these tests it may be stated that alcohol, when added to blood, ground pad tissues, or aspirated lymph containing the virus, causes the formation of a more or less dense coagulum. Centrifugation of these agglomerations leaves a supernatant fluid which is still active. We have already shown that the virus as it occurs in the tissues of the guinea pig is not centrifugable and we have discussed the inability of the centrifuge to depose or sediment completely minute particles.[1] On the other hand, filtration is a more effective means of separating particles from a fluid. Hence by employing Berkefeld filters, which owing to their relatively larger sized pores permit the passage of the active agent irrespective of electric charge,[2] these particles are thereby withheld from the filtrate. The filtrate then is free from virus. In other words, the active agent is closely attached or adsorbed to or forms a nucleus in the center of the coagula formed after the addition of alcohol.

Similar results were obtained with acetone. In pure acetone, which forms a heavy soft coagulum, the active agent survived for 20 but not for 30 minutes. If the material was filtered immediately after complete coagulation occurred, the Berkefeld filtrate was inactive.

With ammonium sulfate the result was the same. In this instance, materials containing the virus after supersaturation with ammonium sulfate for 1 hour at 37°C., exhibited a heavy coagulum. The latter was active but on filtration of the substance through Berkefeld candles, the filtrates were inactive. In two instances the clear filtrate obtained by employing 2 layers of Schleicher and Schüll, No. 589 filter paper was also free from virus.

Hence it appears that the active agent in guinea pig tissues is completely adsorbed, and protected, as we shall soon show again,

by the precipitates formed after the addition of alcohol or acetone or ammonium sulfate.

The next step to suggest itself in a further study of this phenomenon was the relation of the amount of protein in the material containing the virus to its capacity for resisting the action of the reagents.

By filtration of vesicular fluid through Berkefeld candles, it was possible to remove a considerable amount of protein substances, especially cells, fibrin, clots, and larger miscellaneous particles. Filtrates, however, usually showed the biuret reaction so that not all proteins were thus eliminated. However, when alcohol or acetone was added to filtrates, only a faint haze of micellæ resulted. How this influences the resistance of the virus to the reagents is illustrated in the following experiment.

Protocol 2.—1 cc. of aspirated lymph from pads injected 24 hours previously was added to 20 cc. phosphate buffer at pH = 7.5. This was then filtered through a Berkefeld N candle and of the filtrate 5.4 cc. were taken. To this amount, 10 cc. of 90 per cent alcohol were added which made the concentration of the latter equal to 60 per cent. 0.1 cc. of the remainder of the filtrate, non-alcoholized, was injected into each of 2 guinea pigs, as controls, to test its activity. In 48 hours both animals exhibited the typical experimental disease.

A series of guinea pigs was now inoculated intradermally with the alcoholized filtrate after varying intervals: After 5 minutes, only 1 of 2 animals injected was positive with an incubation period of 4 days; after 15 minutes the same result occurred with an incubation of 5 days; after 30 minutes and 1 hour, the filtrate proved inactive.

This experiment is typical of several others in some of which 50 per cent alcohol as well as filtrates of ground infected pad tissues were also employed.

The conclusions to be derived from all these tests are that although the unfiltered virus remained viable in 50 or 60 per cent alcohol for at least 26 hours, filtered material showed no such resistance: When 50 per cent alcohol was added to active filtrates of aspirated lymph or ground infected pad tissues, the virus was killed within 15 to 20 minutes, and in 60 per cent alcohol, within 1 to 15 minutes, depending on the concentration of the active agent. The unfiltered material contained more protein substance than the filtered and hence a greater amount of coagulum which displayed a greater ad-

sorptive function. The consequence was that the virus in filtrates was more open to the direct attack by the reagent.

As a corollary, the immediate refiltration of the active coagulum formed by adding alcohol to filtrates, removed the virus. This is not due to double, or repeated, filtration of the virus materials, for control tests showed no such tendency.

From these observations further proof is adduced that there is a relationship between the amount of virus adsorbed to large coagula and the killing effect of alcohol. There is an indication here which points to the possibility of increasing the destructive action of alcohol, if the latter could be placed in contact with free, or unadsorbed, virus. We have already stated[1] that the virus can exist in a free state.

We believe that we have succeeded in bringing this about by taking into consideration the fact that coagulation of proteins by alcohol is a periodic phenomenon, depending on hydrogen ion concentration.[6,7] For example, a basic experiment was performed with ascitic fluid as the protein material. It was found that the heaviest coagulation by 60 per cent alcohol occurred between pH = 7 and 7.6, maximum at pH = 7.5. At pH = 8.2 there was absolute clearing and at pH = 6.5, only a slight micellar haze was noted. Hence at a certain point, reached by adding a definite minute quantity of 0.5 per cent NaOH to protein material, alcohol does not coagulate. Furthermore, the quantity of alkali necessary to prevent this coagulation is insufficient *per se* to kill the virus. Since it is impossible to make determinations of the hydrogen ion concentration of alcohol, we proceeded in an empiric manner, after the method of Loeb[8] in his studies on solutions of protein in alcohol-water mixtures, to determine precisely the amount of alkali necessary to inhibit coagulation.

Protocol 3.—A large number of experiments was made over a period of 3 months and several hundred guinea pigs were employed before unequivocal results could be

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[6] Compare Michaelis, L., Manuel de techniques de physico-chemie, translation by Chabainer, H., and Labo-Onell, C., Paris, 1923, 81 ff., or the original in German.

[7] Kopaczewski, W., Théorie et pratique des colloïdes, Paris, 1923.

[8] Loeb, J., Proteins and the theory of colloidal behavior, New York, 2nd edition, 1924, 359 ff.

obtained. It is only possible to state here a résumé of results, and of the conditions necessary for procedure.

We found these technical operations to be important in carrying out the tests.

1. All material should be measured in conical beakers or chemical mixing glasses and not in test-tubes, for spattering of the virus or insufficient mixing of the substances may give irregular results.

2. The active virus, whether in aspirated lymph or ground infected pad tissues should be filtered first through filter paper to remove clots and small particles. The dilution employed in tests and controls was 1:50 to 1:100. In each experiment, the paper filtrate was injected intradermally in guinea pigs to test its activity.

3. The phosphate buffer[9] at pH = 7.5 should be adjusted to this point just before use, since sterilization or standing in glass utensils may cause a change in hydrogen ion concentration. The buffer is used only for making suspensions of the virus. For controls (see Table I) distilled water is substituted for the alcohol or NaOH as the case requires so that the buffer effect remains constant throughout each series.

4. In the test, the alkali should be added first, then the alcohol (for water in controls), and lastly the virus. Immediately thereafter these ingredients are thoroughly mixed, and after the time set for contact, fluid is removed from about the centre of the mixture.

5. Since the measure of the killing effect of alcohol is not cultural but biological, that is, the injection of the test materials intradermally in guinea pigs, great care should be observed in differentiating the dull white alcoholic, or the dirty yellow alkali, inoculation necroses, from the lesions of experimental foot-and-mouth disease. As safeguards it was necessary, (*a*) to have a strain of virus which produced regularly secondary vesicles, (*b*) to transfer suspected tissues to normal guinea pigs for the observation of uncomplicated effects, and (*c*) whenever possible to subject recovered animals to an immunity test.

In the tests 60 per cent, 40 per cent, and 20 per cent alcohol dilutions were employed. These percentages indicate the final concentrations which were obtained by using stock alcohol of 90 per cent strength. Of this a sufficient amount was taken to make the required concentration, in a total volume of test or control materials of 7.7 cc.

It was found that in this volume and with these percentages of alcohol the quantity required of the NaOH, 0.5 per cent solution, was 0.33 cc.

In Table I, the different amounts of the materials employed are tabulated, as well as the general manner of procedure, both in regard to the tests and their corresponding controls.

From this tabulated résumé it is to be noted that the active agent as it exists in the tissues or exudates of the guinea pig resists the action

[9] For its preparation, Papers I and II should be consulted.

of alcohol in dilutions of 60, 40, or 20 per cent. But the resistance is due to the adsorbing coagulum which results from the interaction of alcohol on the proteins of the milieu in which the virus resides. The latter is also unharmed by the presence of NaOH 0.5 per cent in the

TABLE I.

Effect of Alcohol on Virus.

Time	For 60 per cent proportion			For 40 per cent proportion			For 20 per cent proportion		
	Control	Control	Test	Control	Control	Test	Control	Control	Test
	Virus 2.7 cc. + alcohol 90 per cent 5 cc. + H_2O 0.33 cc.	Virus 2.7 cc. + H_2O 5 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 2.7 cc. + alcohol 90 per cent 5 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 4.4 cc. + alcohol 90 per cent 3.3 cc. + H_2O 0.33 cc.	Virus 4.4 cc. + H_2O 3.3 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 4.4 cc. + alcohol 90 per cent 3.3 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 6.0 cc. + alcohol 90 per cent 1.7 cc. + H_2O 0.33 cc.	Virus 6.0 cc. + H_2O 1.7 cc. + NaOH 0.5 per cent 0.33 cc.	Virus 6.0 cc. + alcohol 90 per cent 1.7 cc. + NaOH 0.5 per cent 0.33 cc.
1 min.....	+	+	−	+	+	−	+	+	+
2½ hrs.. ...	+	+		+	+		+	+	−**
6 "	+	+*		+	+		+	+	
24 "	+			+	+*		+	+*	
26 "	+			+*			+*		
30 "	−								

+ = virus active, − = virus killed.

* 6 hours was the longest time tested. But 0.27 cc. of NaOH, 0.5 per cent, in a 7.7 cc. suspension did not kill the virus after 24 hours.

** Average time of 5 experiments.

small quantity used, that of 0.33 cc. to 8.03 cc. (or about 1 in 5000) of the medium. But in the presence of the alkali, alcohol fails to coagulate the protein: the resultant mixture is clear. Then the alcohol comes into direct contact with the unprotected virus and kills

it quickly; the 40 per cent and 60 per cent dilutions within 1 minute, and the 20 per cent, within an average time of 2½ hours.[10]

We could make no comparison with other filter-passing viruses in respect to sensitiveness to alcohol under similar conditions. Hence we turned our attention to a definite living microorganism, the staphylococcus, for a relative study.

Comparison with Staphylococci.—In the following experiments cultures of *Staphylococcus aureus*, obtained from a patient with osteomyelitis, and grown on plain agar slants were substituted for the virus materials, and then subjected to alcohol action under similar conditions.

Protocol 4.—Suspensions of staphylococci were made in phosphate buffer at pH = 7.5. A proportional amount of this and of the stock 90 per cent alcohol was taken to make final concentrations of 60, 40, and 20 per cent of the latter, with the same quantities outlined in Table I for the virus-alcohol tests. This constituted 1 series, to check the next in which again 0.33 cc. of NaOH 0.5 per cent was added to the volume of 7.7 cc. of staphylococcus-alcohol suspensions. A tabulation of the results is given in Table II.

In respect to a suspension of staphylococci containing only microorganisms which are not admixed with proteins as is the case with the virus, Table II shows that 60 per cent alcohol was capable of complete inhibition of growth within 1 minute. 40 per cent alcohol killed staphylococci after 6 minutes and 20 per cent after 3 days. But with this microorganism as well, the addition of the alkali increased somewhat its sensitiveness to the reagent: in 40 to 60 per cent alcohol the lethal action was too short to make a definite difference but in 20 per cent alcohol the killing time was shortened 2 days.

We may now make a comparison between the virus and staphylococci in regard to this phenomenon.

In 60 per cent alcohol the virus in its protein milieu remains alive for at least 26 hours—for a much longer time according to Abe and the workers of the British Commission. But freed from the protective coagulum, the virus is killed within 1 minute—so are staphylococci.

[10] In 4 instances the lethal point was 1½ hours, 1 hour, or less, but in 1 instance which we cannot explain, the virus resisted 20 per cent alcohol plus NaOH for 7 hours. Stockman and Minett,[4] however, found that 25 per cent alcohol *per se* kills the virus after 3 days and 10 per cent after 20 days.

In 40 per cent alcohol, the virus in the guinea pig tissues or exudates is resistant for at least 26 hours, or even several days. In its free state, however, it is killed also within 1 minute. The growth of staphylococci is inhibited, on the other hand, after $5\frac{1}{2}$ to 6 minutes.

TABLE II.

Action of Alcohol on Staphylococci.

Time	Staphylococci + alcohol			Staphylococci + alcohol + NaOH 0.5 per cent 0.33 cc.		
	60 per cent	40 per cent No. of colonies	20 per cent alcohol No. of colonies	60 per cent	40 per cent No. of colonies	20 per cent alcohol No. of colonies
min.						
1	—		Inf.	—	10	Inf.
$1\frac{1}{2}$		110	"			"
$3\frac{1}{2}$			"		5	"
$5\frac{1}{2}$			"		2	"
6		2	"		—	"
$6\frac{1}{2}$		—	"			"
60			"			"
hrs.						
$2\frac{1}{2}$			"			"
4 to 7			"			P. G.
8 to 22			P. G.			" "
23			" "			10
24			" "			—
days						
2			75			
3			8			
4			—			

— = no growth in subculture of 0.2 cc. Inf. = prodigious, normal growth as compared with controls. P. G. = profuse, coalesced growth making it impossible to count individual colonies.

Control cultures of the staphylococcus suspension showed the usual growth after 4 days.

In 20 per cent alcohol the virus in its natural state can remain viable for at least 3 days.[4] But when it is not adsorbed to coagula and there exposed directly to the action of this reagent it is killed in from less than 1 to 7 hours (in an exceptional case). Staphylococci,

on the other hand, are killed after 23 hours (with alkali) or after 3 days (without alkali).

In conclusion it may be stated that in testing viability of the virus admixed with proteins, one encounters complications due to the protective action of the protein coagula. When coagulation is prevented by taking advantage of the periodic phenomenon, and the virus is exposed *in toto* to the action of the chemical, it is then even more sensitive to destruction than a culture of a known microorganism, such as staphylococcus. The view-point, therefore, that the active agent of foot-and-mouth disease, by itself, is more resistant to alcohol than living bodies, such as bacteria, is incorrect.

Alcoholized Virus in Cultures.—It was believed that concentration of the virus could be effected by its adsorption to the coagulum which is formed by adding 60 per cent alcohol to the active agent in a protein milieu. By employing such "alcoholized" virus we also had in mind the possibility of removing ordinary, contaminating bacteria and, at the same time, the inhibiting substances which prevent growth *in vitro*. Results of experiments show that the virus prepared in this manner cannot be used for culture: for example, in buffered gelatin (described in the next paper on cultivation experiments), control virus survived at 34°C. for at least 9 days but no activity was noted with alcoholized virus on the 5th day of incubation.

Virucidal Chemicals.

It may be concluded from the observations already given that chemicals employed as antiseptics, which produce coagulation of proteins and lead to protection of virus adsorbed to the formed particles, may not be active as virucides. On the contrary, such substances which do not coagulate the proteins of the material containing the virus, thus placing one in more direct contact with the other, may act more powerfully as destructive agents. As the following will show we were justified in this belief.

The scope of this test was extended to include chemicals which are coagulating and those which are not, employing with both the same sample of virus materials and comparing their powers of destruction. Accordingly we chose as examples of the 1st group, such substances as bichloride of mercury and cresol, 2 samples. Later a

new preparation advanced as a certain antiseptic against the foot-and-mouth disease virus, chloronal,[11] was added. As specimens of the 2nd group of non-coagulating substances we selected antiformin and sodium hydrate.

In view of the fact that under actual field conditions coagulants are generally employed as virucides, most often cresol and bichloride of mercury, the results of the following experiments take on a greater significance.

The 1st experiment dealt with the virucidal properties of bichloride of mercury and 2 different samples of cresol, one labelled as such, and the other, as a cresol compound. Protocol 5 illustrates one such test:

Protocol 5.—Aspirated lymph virus was diluted in phosphate buffer at pH = 7.5. This form of virus was employed instead of fragments of infected tissue so as to favor any destructive action of the chemicals. To 2 cc. of 1:100 of the suspension of the active agent were added 2 cc. of 1:500 bichloride of mercury and to other respective lots, 2 cc. of 6 per cent cresol and *liquor cresolis compositus*. In the end the dilution of the virus was 1:200, of the bichloride 1:1000, and of the cresol preparations 3 per cent. After varying periods of contact, from 5 minutes to 6 hours (the longest time of testing), the mixtures were injected intradermally in guinea pigs. The results with all these substances were practically identical: Guinea pigs injected with material after 5 minutes to 2 hours contact showed primary vesicles in 24 hours and secondary lesions in 48 hours. Those animals injected with material after 4 to 6 hours contact exhibited primary lesions in 48 hours and secondary vesicles after 72 hours.

All the precautions mentioned in Protocol 3 were employed and great care was taken not to confuse chemical necroses with the lesions of experimental foot-and-mouth disease.

Hence bichloride of mercury, cresol, or compound cresol, all forming large coagula with virus materials, act as does alcohol. They do not destroy the virus quickly, at least, not after 6 hours, the longest period tested. On the other hand, in 2 other experiments chloronal in a 5 per cent solution, which substance forms much lesser and finer coagula, failed to destroy in 30 to 32 minutes but after 1 hour inactivated the virus.

[11] Trautwein, K., *Arch. wissensch. u. prakt. Tierheilk.*, 1925, lii, 254. According to this writer, chloronal contains 25 to 30 per cent chlorine, mainly in a dissociable state; but it is stated that it coagulates albumen.

The next step was to test the action of antiformin and sodium hydrate, substances which do not coagulate the proteins of the medium containing the virus.

Protocol 6.—Of antiformin, 1 per cent was employed; of sodium hydrate, ¼, ½, 1, and 2 per cent. The virus was diluted in phosphate buffer to 1:40 and, as a more severe test of the action of these reagents, consisted of ground infected pad tissue, unfiltered, and containing particles up to about 3 mm. in size. In other experiments in this series, aspirated lymph was also used as a source for virus; in still others the latter was diluted only 1:10. In all cases, however, the results were identical, and as follows:

Antiformin 1 per cent in contact with virus for 1, 2, 3½, 5, 15, and 60 minutes. All guinea pigs injected intradermally failed to show signs of the experimental disease.

Sodium hydrate ¼ per cent. 2 of 4 guinea pigs injected intradermally exhibited, in both cases, after 3 days, primary lesions of the experimental disease, with specimens in which the alkali and virus were in contact for 1 minute. The virus, however, after exposure for 3 minutes was inactive.

Sodium hydrate ½ per cent. 1 of 4 guinea pigs injected intradermally showed after 2 days primary vesicles and after 5 days, secondary lesions. Exposure of virus to the alkali 1 minute. After 3 minutes contact all the injected animals remained well.

Sodium hydrate 1 per cent in contact with the virus for 1 and 3 minutes. None of 4 guinea pigs injected were affected.

Sodium hydrate 2 per cent in contact with the virus (1:20 to 1:40 final dilution) for 1 to 3 minutes. Of 10 guinea pigs injected intradermally with the mixtures, all remained normal. Observations were also extended to cover a period of contact from 3 minutes to 1 hour. Again none of the latter injected animals was affected.

It is to be noted that variations in the technique outlined in Protocols 3 and 5 may lead to errors of interpretation. With antiformin and sodium hydrate, especially in higher concentrations, chemical necroses are apt to occur at the site of injection which may mask the primary lesions. Hence attention should be given to the appearance of secondary lesions. In addition, material from all suspected pads should be removed and suspensions thereof injected into a series of normal animals for control of the specificity of the reaction, and finally, whenever possible recovered animals should be tested for immunity.

These experiments demonstrate that chemicals such as antiformin and sodium hydrate, which do not coagulate the proteins of the medium containing the virus and therefore coming in direct contact with the active agent, were powerfully destructive to the incitant.

Antiformin in 1 per cent killed the virus within 1 minute.[12] Sodium hydrate $\frac{1}{4}$ to $\frac{1}{2}$ per cent destroyed the active agent within 3 minutes but not in 1 minute. At this concentration, however, the destructive effect was weak and irregular. But in 1 to 2 per cent solutions, the inhibiting action occurred within 1 minute and was complete and regular. It may be stated in favor of the completeness of this neutralizing effect, that the virus was penetrated and killed even when present in fragments of tissue as in ground infected pad tissues containing particles about 3 mm. long.

The question arose then as to whether soda could replace sodium hydrate in respect to the latter's powerful action. But our experience with 5 per cent anhydrous sodium carbonate has shown that this could not be done.

The regular and energetic destruction of the virus by sodium hydrate in low concentrations led us to seek a practical application. Field conditions were imitated by admixing virus in bits of tissue with cow's urine, with manure, and with garden earth, and then exposing such mixed materials to the action of sodium hydrate. An illustrative experiment, one of several, follows:

Protocol 7.—24 hours after injection, 4 infected pads were removed from guinea pigs and ground in 20 cc. of phosphate buffer at pH = 7.6. To this were added 20 cc. of aspirated lymph virus of a dilution of 1:10. The material was unfiltered and contained a number of small particles of tissue. 2 cc. of this virus suspension were added to 2 cc. of urine from a normal cow. The probable dilution of virus in this instance was 1:20. 2 cc. were also added to about 5 gm. of manure from normal cattle and to a similar amount of earth obtained from an area near the stalls. In all instances the virus was thoroughly admixed with the substances to which it was added and the mixtures were allowed to stand at room temperature for 1 hour.

Then to the virus suspension itself, and to the urine, the manure, and the earth containing the virus, a quantity of sodium hydrate 4 per cent solution was added to make the final concentration of the latter 2 per cent. Guinea pigs were injected intradermally with the mixtures exactly 1, 3, and 5 minutes after adding the alkali. Observations extended over 12 days showed that none of these animals became infected.

[12] Stockman and Minett[4] have also found that the virus (in comparison with its great resistance to alcohol, ether, etc.) is easily destroyed, in "less than 1 hour," by antiformin 1 per cent.

At the end of an hour or after the completion of the test, portions of the virus suspension itself, and the urine, the manure, and the earth containing the virus, were injected intradermally in respective guinea pigs, which served as controls for activity of the different materials. In all animals typical primary lesions were observed 24 hours after injection and secondary vesicles 12 to 24 hours later.[13]

It appears, therefore, that sodium hydrate in a 2 per cent solution is capable of destroying, within 1 minute, not only the virus in its ordinary condition even when contained in fragments of tissue, but also such virus admixed with cattle urine, with manure, and with earth.

As a further application of the virucidal effect of this reagent to field conditions, the test was extended to include cattle as experimental animals. The results are shown in the following protocol.

Protocol 8.—The materials employed in this experiment were prepared in a manner exactly similar to that mentioned in Protocol 7. To portions of a suspension of virus, and virus mixed with cow's urine, with manure, and with earth,—in all cases thoroughly incorporated,—was added sodium hydrate in sufficient quantity so that the final concentration of the latter was 2 per cent (see Protocol 7). 1 minute later 9 cattle were inoculated by the method of scarification of the mucous membrane of the mouth and then rubbing in the substances quite briskly: 3 cattle were inoculated with the NaOH-virus material and 2 each with the remaining 3 mixtures, respectively.

1 hour later 3 normal cattle and 4 guinea pigs were inoculated, the former by the same scarification method employed in the test animals, with the suspension of the virus; and the latter intradermally, with the portions of the remainder of the 4 different virus mixtures. These animals served as controls for the activity of the virus and the latter admixed with urine, manure, or soil. In addition to these controls, a hog was injected intravenously and 2 convalescent heifers locally.

None of the cattle inoculated with the virus mixtures exposed to the action of sodium hydrate for 1 minute was affected, while all of the control animals except the convalescent heifers showed within 48 hours symptoms of experimental foot-and-mouth disease.

The 9 cattle employed in the test series—the 1st group—were inoculated, after 28 days, by the same scarification method, with active virus. All of them became ill with the typical experimental disease; thus demonstrating that the absence of effect from the first inoculation was not due to a natural resistance.

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[13] Care should be taken not to confuse chemical necroses with the true, typical lesions of experimental foot-and-mouth disease. (See Protocol 3, Paragraph 5.)

One may conclude from the foregoing that the destructive effect of 2 per cent sodium hydrate on the virus is complete and rapid—within 1 minute—as shown by tests not only on guinea pigs but on cattle as well. This leads to the consideration of this reagent as an effective virucide in practice.[14]

Comparison of Action of These Reagents on Staphylococci.—We now turned our attention to the action of the different chemicals employed as virucides, upon definite living microorganisms such as staphylococci. The object in such determinations was to note whether the

TABLE III.

Effect of Certain Chemicals on Staphylococcus aureus.

Time	Bichloride of mercury 1:1000	Cresol 3 per cent	Chloronal 5 per cent	Antiformin 1 per cent	NaOH 2 per cent	Control suspension of staphylococci
	No. of colonies					
min.						
1	1000	Inf.	4	14	200	Inf.
3	100	"	1	—	30	"
5	5	"	—	—	5	"
7	—	P. G.	—	—	—	"
10	—	1000	—	—	—	"
15	—	300	—	—	—	"
60	—	26	—	—	—	"
120	—	—	—	—	—	"

The indicating marks are similar to those used in Table II.

active agent of foot-and-mouth disease is by virtue of these reactions in a class by itself, or whether it can be correlated with living bacteria.

Protocol 9.—Suspensions of staphylococci were prepared as in the experiments cited in Protocol 4. To these were added, respectively, bichloride of mercury in 1:1000, cresol and cresol compound, in 3 per cent, chloronal in 5 per cent, antiformin in 1 per cent, and sodium hydrate in 2 per cent, final concentrations. After varying periods of time at room temperature, 0.1 cc. was subcultured on agar. The results are tabulated in Table III.

[14] The limited time and space at our disposal prevented similar tests on cows with 1 per cent sodium hydrate. But the results with guinea pigs demonstrate that this concentration of the chemical may be equally effective.

The results summarized in Table III reveal that pure cultures of staphylococci are killed by contact with bichloride of mercury 1:1000 after 5 minutes; by cresol 3 per cent, after 1 hour; by chloronal 5 per cent, after 3 minutes; by antiformin 1 per cent, after 1 minute; and by sodium hydrate 2 per cent, after 5 minutes.

The destructive action of these substances on pure staphyloccoci may now be compared with that on the virus as it is ordinarily employed in a protein medium. For greater ease of comparison the results of the latter experiments are summarized in Table IV.

TABLE IV.*

Effect of Certain Chemicals on the Virus (1:40).

Time	Bichloride of mercury 1:1000	Cresol 3 per cent	Chloronal 5 per cent	NaOH 2 per cent	Antiformin 1 per cent	Control of virus
min.						
1	+	+	+	−	−	+
3	+	+	+	−	−	+
5	+	+	+	−	−	+
15	+	+	+	−	−	+
20	+	+	+	−	−	+
30	+	+	+	−	−	+
32	+	+	+	−	−	+
hrs.						
2	+	+	−	−	−	+
4	+	+	−	−	−	+
6	+	+	−	−	−	+

* The virus being either in the form of lymph or admixed with, adherent to, or incorporated in, small fragments of tissue. 6 hours was the longest period tested.

Table IV shows that the virus resisted the chemicals which caused protective coagulation of the proteins of its medium, but was killed within 1 minute by those which did not form a coagulum. On comparing the results given in Table III with those in Table IV, it will be noted that the active agent when not protected by coagula is still more sensitive to destruction by the same reagents, sodium hydrate and antiformin, than are staphylococci. Hence these tests confirm the prior conclusions with respect to the action of alcohol on the virus and on staphylococci. Furthermore, none of the effects of

these reagents on the virus can be interpreted as an indication of its inanimate character.

SUMMARY AND CONCLUSIONS.

The virus of foot-and-mouth disease exhibits a remarkable resistance to such bactericidal agents as the narcotic solvents (alcohol, ether, chloroform), or such antiseptics as phenol, bichloride of mercury, or cresol, as shown by tests made by others and ourselves. We have shown, however, that the resistance of the incitant to these chemicals is really masked. It is due to the fact that the reagents coagulate the proteins of the medium in which the virus is, as a rule, suspended. As a result the active agent is protected by the coagula which prevents direct contact with the chemicals. On the other hand, if advantage is taken of the periodic phenomenon attending such processes, and coagulation is prevented, the virus can then be brought under direct action of the antiseptics. Under these conditions, the incitant is more sensitive to destruction by the chemicals than is the living staphylococcus. As a corollary, the virus is destroyed as rapidly, or even more so, than are staphylococci by substances such as sodium hydrate (1 to 2 per cent solutions), or antiformin (1 per cent solution) which do not form coagula. We are therefore compelled to contradict the opinion that the extraordinary resistance to certain chemicals of the virus of foot-and-mouth disease, as it ordinarily occurs admixed with proteins, is an indication of its inanimate character.

The results of a large series of experiments lead to the conclusion that of a number of antiseptics employed the sodium hydrate in 1 to 2 per cent solutions is an effective virucide. It is capable of killing the virus within 1 minute as shown by tests on cattle and guinea pigs. Furthermore, its effectiveness is not diminished even when the virulent material is admixed with cattle's urine, with manure, or with garden soil. The experimental evidence and the cheapness suggest its use in field practice as a disinfectant.

STUDIES ON THE PHYSICAL AND CHEMICAL PROPERTIES OF THE VIRUS OF FOOT-AND-MOUTH DISEASE.

IV. Cultivation Experiments.

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(Received for publication, December 22, 1926.)

In the first paper[1] of this series we stated that the lesions induced by the foot-and-mouth disease virus are free from ordinary bacteria. But before undertaking an extensive study on cultivation, it was believed desirable to add to the routine morphological studies and to the use of some ordinary means of culture, a large variety of media under different atmospheric conditions, different temperatures, and with different sources of the active agent. In this way we thought that there might be revealed any constant, secondary or concomitant, microorganism.

Accordingly, a large number of experiments were made with the virus in the blood of actively infected guinea pigs, with filtered and unfiltered vesicular contents, and with filtered or unfiltered ground pad or foot tissues from guinea pigs or cattle. The materials were cultured in media at room temperature and at 32–37°C. The atmospheric conditions varied from aerobic to anaerobic and in either condition plus the addition of carbon dioxide (up to 25 per cent concentration). A variety of media, both fluid and solid, was used: ascitic fluid with 5 per cent guinea pig, defibrinated blood; ascitic fluid, calf tongue broth; 5 per cent sheep blood, 1 per cent dextrose calf tongue broth and these media with 2 per cent agar. Agar plates with rabbit or sheep blood were also employed.

No constant, visible microorganisms were detected in these artificial media, even after repeated subplants. Nor, in the absence of visible

[1] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 673.

growth, were we enabled to obtain a pathogenic culture sufficiently diluted to eliminate actual transfer of the virus. These preliminary tests showed, therefore, that the ordinary methods appeared to be unsuitable for the cultivation of the virus. Another conclusion was that only the filtered pad material and the blood were useful for cultivation: the unfiltered ground infected pad tissues or aspirated "lymph" were often admixed with the different bacteria normally present in the pads of guinea pigs or in the mouths of cattle, and the usual method of searing the surface was ineffective.

Our plan of study from this point was to make a deductive inquiry into the conditions which were best suited for the viability of the active agent *in vitro*. It was thought that this way of approach would yield more satisfactory results in our limited time than that of applying at once special methods of cultivation. But before this could be done, it was essential to investigate the findings of Frosch and Dahmen.[2]

A widespread interest was aroused in 1924 when these writers announced the cultivation by special methods, of minute bacillary bodies, *Löffleria nevermanii*, which they maintained were the inciting agents of foot-and-mouth disease. Their colonies, as well as the bodies themselves, were visible only by means of short wave-length, ultra-violet, photomicrography. Their method of culture consisted in centrifuging the virus materials (lymph) over a long period to concentrate the virus, and then washing the sediment in changes of salt solution to free the virus from inhibiting substances. The sediment was seeded on a solid, slope medium containing Martin broth, 3 per cent agar, to which was added, just before use, 20 to 50 per cent of horse or ox serum. The medium was adjusted to pH = 7.8. Growth occurred aerobically after 7 to 10 days at 33–34°C. Suspensions of these subvisible colonies, even to the 25th generation, induced in guinea pigs experimental foot-and-mouth disease, albeit at times the reactions were quite feeble.

In accordance with the statements of Frosch and Dahmen, prolonged centrifugation of the virus materials was a prerequisite for successful cultivation. We have already shown (*vide supra*) that this step in the technique does not concentrate the virus, nor can the so called inhibiting substances be thereby removed. Furthermore, by repeated tests the virus failed to survive in the Frosch and Dahmen medium after 3 days, although with the same specimen of virus and seeded at

[2] Frosch, and Dahmen, H., *Arch. wissensch. u. prakt. Thierheilk.*, 1924, li, 99, 108; *Berl. tierärztl. Woch.*, 1924, xl, 185, 273, 341, 662.

Dahmen, H., *Berl. tierärztl. Woch.*, 1925, xli, 733.

the same time, it remained viable for 7 days in buffered 10 per cent gelatin at pH = 7.6 and for 9 days in buffered phosphate[3] at pH = 7.6. We will show later that broth is an unsuitable medium; this applies with equal force to the serum. In addition, the hydrogen ion concentration and the aerobic conditions which are employed in this method were also found unsatisfactory.

We could not confirm the conclusions of Frosch and Dahmen, nor were we favored by finding therein, on analysis, any clew which might lead to a lengthening of the life of the virus under artificial conditions. In respect to our lack of confirmation of Frosch and Dahmen's results we are in agreement with the German Commission directed by Dr. Gins,[4] who also found that in Martin broth serum agar the virus dies in 3 days. We are also in accord with the work of the British Commission.[5-7] These investigators have shown that the so called colonies and their constituents could be imitated by materials other than the virus of foot-and-mouth disease.

In view of the importance generally ascribed to the hydrogen ion concentration of media in affecting growth or viability of microorganisms, our earlier studies were directed to the optimum range required for the virus.

Optimum Hydrogen Ion Concentration.—As the following illustrative protocol shows, variation in the hydrogen ion concentration of the media containing the virus affected its viability.

Protocol 1. The same specimen of virus, 24 hour old aspirated lymph in phosphate buffer and filtered through a Berkefeld candle, was employed in all these tests. ½ cc. was inoculated into each 10 cc. of the medium to be stated.

(*a*) Buffered 10 per cent gelatin at pH = 6.9 after 5 days' incubation at 34°C. 2 guinea pigs injected intradermally. Both negative.

Buffered 10 per cent gelatin at pH = 7.5 after 5 and 7 days' incubation at 34°C. 4 guinea pigs injected, 2 each respectively with the 5 and 7 day incubated materials. All positive.

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[3] For its preparation Papers I and II should be consulted.

[4] Gins, H. A., *Berl. tierärztl. Woch.*, 1924, xl, 661; abstracted in *J. Comp. Path. and Therap.*, 1924, xxxvii, 302.

[5] First Progress Report of the Foot-and-Mouth Disease Research Committee, Ministry of Agriculture and Fisheries, London, 1925.

[6] Arkwright, J. A., Burbury, M., Bedson, S. P., and Maitland, H. B., *J. Comp. Path. and Therap.*, 1925, xxxviii, 229.

[7] Stockman, S., and Minett, F. C., *J. Comp. Path. and Therap.*, 1926, xxxix, 1.

(*b*) Buffered agar ½ per cent at pH = 8 after 3 days' incubation. 2 guinea pigs injected intradermally. Both negative.

Buffered agar ½ per cent at pH = 7.6 after 3, 5, 7, and 9 days' incubation. 8 guinea pigs injected, 2 each respectively with a portion of each specimen. All positive.

These instances do not show the extreme effect of variations of hydrogen ion concentration on the preservation of the virus: in 1 case in 2 per cent dextrose, 5 per cent sheep blood, calf tongue extract medium the latter survived 25 days longer at room temperature at pH = 7.4 than at pH = 6. In general, a large experience demonstrated that the optimum hydrogen ion concentration best suited for the viability of the active agent *in vitro* is at pH = 7.5 to 7.6. Variations above or below this narrow range exhibit an unfavorable influence. We are thus in agreement with the findings of Stockman and Minett[7] and of Bedson and Maitland[6] of the British Commission. Conversely, it became apparent from a number of tests which need not be quoted here, that the phosphate buffer solution at pH = 7.5 or 7.6 could be employed to greater advantage as a base for the preparation of a wide variety of media used in tests of their preserving action, than media not prepared on this base.

Temperature.—The active agent is very sensitive to higher temperatures. We have already stated that the virus in guinea pig blood survives after 24, but not 48 hours at 37°C. The following protocol exemplifies the advantageous effect of lower temperatures.

Protocol 2.—Virus material consisted of a 1:100 dilution of aspirated lymph in phosphate buffer, adjusted to pH = 7.6 and filtered through a Berkefeld candle. One portion was kept at 37°C., another at 32–34°C., and a third at 18–20°C. (room temperature). After varying intervals of time, the materials were injected intradermally in guinea pigs. The virus retained at 37°C. survived for 3 days but not for 5; at 32–34°C. for 9 days but not for 11; and at 18–20°C. for at least 25 days. In addition, in this medium plus 50 per cent glycerol the virus remained alive indefinitely in the ice box (2–5°C.).

In another test with a more complex medium, the dextrose, sheep blood, calf tongue extract mentioned kept under anaerobic conditions, the virus survived herein for 6 days at 37°C., and for 25 but not for 32 days at 18–20°C.

From these and other observations to be described later it appears that the virus of foot-and-mouth disease like that of mosaic disease[8]

[8] Johnson, J., *Phytopathology*, 1921, xi, 446; 1922, xii, 438.

and other filter passers was sensitive to higher but was quite resistant to lower temperatures. At 37°C. we were unable to keep the active agent alive for more than 6 days. At 33–34°C. viability was maintained for 10 days; at 18–20°C. for from 2 weeks to over 2 months (in an instance to be described later), and at ice box temperature, indefinitely. These observations concur in the main with those of the workers of the British Commission.

Oxygen Tension.—The importance of the question whether the lite of the virus is favored by aerobic or anaerobic conditions was appreciated. Hence a number of repeated tests in a variety of media was made, some of which are exemplified in the following:

Protocol 3.—24 hour old virus in the form of aspirated lymph 1 to 20 cc. phosphate buffer at pH = 7.6 was filtered through Berkefeld candles. Of the filtrate 1 cc. was added to each 10 cc. of the medium which was always placed in Petri dishes so as to give it as much surface exposure as possible either, to the aerobic or to the anaerobic atmosphere.

Medium of 10 per cent gelatin prepared by Loeb's method (to be described later). After 5 days' incubation guinea pigs were injected respectively with aerobic and anaerobic materials. Those injected with aerobic subplant showed after 48 to 72 hours mild primary lesions and 24 to 48 hours later similar secondary vesicles. The others, injected with anaerobic material, exhibited within 24 hours severe primary vesicles and 24 hours later many secondary lesions. After 7 days' incubation, aerobic subplants were inactive; the anaerobic could induce the typical experimental disease after 48 hours. After 10 days' incubation, anaerobic material still showed activity in 1 of 3 guinea pigs.

The same general results were obtained when other media were employed.

These experiments indicate that under strict anaerobic conditions the virus retains its viability at least twice as long as under aerobic conditions. The investigators of the British Commission (Stockman and Minett[7]) report, however, that the state of air tension is of no importance from the view-point of survival. The discrepancy in results may be ascribed to a difference of technique. We have employed not only Petri dishes for the tests but also the Boëz apparatus for producing anaerobic conditions.[9] This mechanism employing vacuum, consists of a central post catalyzing station for the combustion of oxygen. It is leak-proof and establishes a perfect and perma-

[9] Boëz, L., *Compt. rend. Soc. biol.*, 1925, xciii, 1663, 1666; 1926, xcv, 708; *J. Bacteriol.*, 1927, xiii, 227.

nent anaerobic atmosphere within a few minutes. None of the methods employed by the British, such as capping tubes, or using a vacuum (Geryk) pump, or exhausting air with or without a hydrogen atmosphere, or boiling, then quickly cooling the medium, and later adding vaseline is comparable to the Boëz apparatus in promptness and effectiveness.[10] Indeed, it may be questioned whether a strict anaerobic condition was ever secured by the British methods.

Influence of Different Media.—The object of the following experiments was to study the effect of different media upon the duration of life of the virus. We began with a series of substances which comprised the Frosch and Dahmen medium, namely broth, serum, agar, and also Witte and Martin peptones.

A. Broth.—We have already stated that the virus died within 3 days in Martin's broth. But no greater success was obtained after repeated trials, with plain broth, buffered broth, tryptic broth, broth prepared with calf tongue instead of beef infusion, and beef infusion itself. In view of the fact that in other media, such as simple buffer phosphate or 10 per cent gelatin, the virus survived much longer under the same conditions of temperature (34°C.) and in parallel experiments, it is apparent that broth is noxious to the active agent. Furthermore, when materials, for example, 10 per cent gelatin, which in themselves were capable of maintaining the life of the virus for a considerable period, were added to broth, not only was the latter unimproved but the favorable action of the intended adjuvant was also lost. As, for example, in 10 per cent gelatin broth the virus did not survive after 3 days, but in the gelatin itself it remained viable after 9 days.

Hence broth including the Martin variety may be considered not only as an unfavorable medium but, moreover, as a rapidly destructive agent to the virus.

The question naturally arose as to what part the peptones of the broth played in this regard. They were therefore put to test.

[10] It was determined, for example, that *Bacterium pneumosintes*, a strict anaerobe, failed to grow by methods depending on the mechanical extraction of air *per se*. In regard to the method of the vaseline seal, the cooling of the medium permits a prompt reabsorption of air (see Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51).

B. Peptones.—2 samples of peptone were available for study: Martin's and Witte's. The following protocol, presenting 1 of many experiments, exemplifies their action.

Protocol 4.—Aspirated lymph, 24 hour old virus was diluted 1:50 in phosphate buffer at pH = 7.5. This was filtered through a Berkefeld candle and 1 cc. of the filtrate was added to 10 cc. of each of the media to be stated. All were incubated at 34°C.

Medium A comprised phosphate buffer at pH = 7.5.

Medium B, the same plus 1 per cent Martin's peptone, and

Medium C, the phosphate buffer plus 1 per cent Witte's peptone.

After 3 days' incubation portions of each were inoculated intradermally into each of 2 guinea pigs, respectively. Within 48 hours all animals showed the typical primary and secondary lesions.

After 5 days' incubation, Medium A could induce primary lesions within 24 hours; Media B and C, much weaker reactions after 48 to 72 hours.

After 7 and 9 days' incubation, Medium A was still active in both of the 2 guinea pigs; Media B and C, inactive.

In addition to this experiment, another was made in which 1 per cent Martin's or 1 per cent Witte's peptone was added to buffered agar or buffered washed 10 per cent gelatin, thus making 4 distinct combinations.

The virus remained alive in the agar (0.25 per cent) for 9 days but in the agar with Witte's peptone for only 5 days and in the agar with Martin's peptone for only 3 days. The virus survived in the 10 per cent washed gelatin for 7 days but in the gelatin with Martin's or Witte's peptone for 5 days. In the latter instances only 1 of 2 guinea pigs showed a feeble reaction after injection.

This experiment shows that Martin's and Witte's peptones are not only unfavorable for the preservation or viability of the virus but that they also tend to exert a harmful effect when added to media which by themselves are favorable to preservation. In this action a counterpart is found in broth. Peptone is one of its constituents which is noxious. Since, however, the period of survival in peptone is somewhat longer than in broth, it appears then that besides it another harmful agent may be present. We have not as yet been able to identify this substance.

C. Serum.—What has been stated for the unfavorable effects of broth or peptone on the survival of the virus applied with equal weight to fresh animal serum and also to human ascitic fluid. For example, the virus remained alive in buffered 10 per cent washed gelatin for 7 days at 34°C., but in this medium with 50 per cent fresh

sheep serum, the same sample of virus was inactive after 5 days of incubation. Repeated tests have shown that fresh sheep or horse serum, or ascitic fluid, added to such substances as agar or gelatin, which by themselves are favorable to the preservation of the virus, exert a virucidal effect. Serum or ascitic fluid also does not counteract the injurious action of broth. Hence these materials are valueless as adjuvants in artificial cultures.

D. Agar.—3 per cent agar is the final ingredient of the Frosch and Dahmen medium. We have found that 2 to 3 per cent agar has an unfavorable influence on the virus for the latter does not survive in this medium longer than 3 days while in other media under the same conditions it survives for a much longer period. However, we extended our studies on the influence on lower concentrations of agar with the hope that perhaps a semifluid, rather than a solid medium might be favorable. The next protocol gives the results obtained.

Protocol 5.—The virus employed in these tests consisted of aspirated lymph diluted 1:30 in phosphate buffer at pH = 7.6 and filtered. 1 cc. of the filtrate was added to 10 cc. of each of the following specimens of agar. The agar was thoroughly washed in distilled water, buffered by the phosphate buffer to pH = 7.6, and prepared in concentrations of 0.1 per cent, 0.25 per cent, and 0.5 per cent. After 5, 7, 9, and 11 days of incubation at 34°C., a portion of each of the inoculated agars was injected intradermally in at least 2 guinea pigs, respectively, for each test. The virus survived in the 0.1 per cent and 0.5 per cent agar for 7 but not for 9 days, but in the 0.25 per cent agar for 9 but not for 11 days.

It is of interest to note that the optimum was at 0.25 per cent of the concentrations of buffered, washed agar employed. In this simple medium the virus survived for 9 days. But as we have already stated, the addition of 1 per cent, Witte's or Martin's peptone, 50 per cent beef maceration, 50 per cent fresh sheep serum, or 50 per cent ascitic fluid, shortened the viability period to 5 days or less. In addition further tests were made with 1 per cent lecithin, 1 per cent dextrose, 4.6 per cent glycerophosphate, and 2 per cent sodium citrate. When these latter substances are added, respectively, to the 0.25 per cent agar, the life of the virus is again shortened to less than 5 days.

It appears from the foregoing, furthermore, that the survival of the active agent depends on the simplicity of the medium in which it is contained—a principle amply confirmed by later tests.

The next step was to test the survival of the virus in 0.25 per cent agar at a lower temperature, namely at 18–20°C.

Protocol 6.—The virus consisted of a filtrate of infected pads, removed 24 hours after injection, ground in phosphate buffer at pH = 7.5. The filtrate, the final dilution of which was 1:100, was injected intradermally in 2 guinea pigs which promptly reacted with the typical primary and secondary vesicles. (Although not mentioned in the prior protocols, this routine procedure was always carried out.) Thoroughly washed agar 0.25 per cent and buffered with the phosphate buffer to pH = 7.5 comprised the medium. To each 10 cc., 1 cc. of the filtrate was added, and the mixtures were kept at 18–20°C. At intervals of 7, 12, 18, 24, 35, 52, and 76 days, portions of this semisolid medium were injected intradermally in at least 2 guinea pigs. It was found that the specimens incubated for from 7 to 52 days were active in the animals but that of the 72nd day, inactive.

Subplants were made from the 1st series of inoculated media after 7 days' incubation into fresh agar. This 2nd transfer also showed activity. The 2nd transfer after 6 days' incubation was subplanted to fresh similar medium. The 3rd was still active. The 4th subplant, however, failed to affect guinea pigs. This test of subplanting the virus was repeated after different periods of incubation and the results were always the same: activity was shown in the first 3 transfers but not so in the 4th—with the proviso that the 3rd transfer was within 52 days after the original seeding of the medium.

In these tests we also encountered a minute bacilloid microorganism which was filter-passing through Berkefeld "V" filters in the 1st but not in later generations. It also grew in peptone and beef maceration semifluid agar. In transferring this microorganism, an admixture of it with some of the virus medium was employed, for it was impossible to obtain a pure culture free from virus. Material containing this microbe was also infectious in the 3rd subplant but not in the 4th.

From the foregoing one may conclude that 0.25 per cent agar is a favorable medium for the preservation of the virus, at least, it is not toxic to it, for it survived in this medium for 52 days at 18–20°C. In view of our later experiences with gelatin which acted still more favorably than agar, it appears that the beneficial effect of the latter is due to its physical property of providing a semisolid structure to the medium.

This experiment also demonstrates that activity in 3 successive subplants was obtained not only with the virus but also with a microorganism for which we have sufficient evidence to indicate that it is merely concurrent with the particular sample of virus studied, and cannot be identified as the active agent itself. But the 4th subplant

in both these instances failed. Since the 4th transfer is the critical one (as explained in Paper I of this series[1]), for in this the virus is diluted beyond its original limit of activity, the infectivity of both the virus and the microorganism—which was maintained not in pure culture but admixed with the virus in the medium—can be interpreted as an expression of preservation but not of multiplication.

E. Gelatin.—The advantage of 10 per cent gelatin, which forms a semisolid medium, over other substances as a preservative of the virus soon became apparent and we were thus led to an extensive study of this material.[11]

It is well known that gelatin contains, as impurities, metals such as arsenic and copper and a considerable concentration of acids. A comparative test was made first with the same sample of virus added to the ordinary Poulenc 10 per cent gelatin and this substance thoroughly washed in distilled water. Both media were buffered at pH = 7.6. The active agent survived in the unwashed gelatin for about 3 days but in the washed, 7 days.

Our next effort concerned the probability of prolonging the preserving action of the washed gelatin by adding to it a number of organic or protein substances:

Protocol 7.—To different lots of 10 per cent washed gelatin prepared on the phosphate buffer base at pH = 7.6 were added, respectively, calf tongue broth, an equal part; Martin's, tryptic, or Witte's peptones, in 1 per cent concentrations; aspargine 0.5 per cent; dextrose 2 per cent, or fresh sheep serum, an equal part. As a control medium, buffered washed agar 0.25 per cent was employed. To each of these substances was added 24 hour old aspirated lymph virus diluted 1:30 in the phosphate buffer, pH = 7.5, and filtered. After varying periods of incubation at 34°C., portions from each were removed and injected intradermally in guinea pigs. The virus survived in the agar for 9 days, in the washed gelatin for at least 7 days, but in the mixtures of organic and protein substances with the washed gelatin, not longer than 3 or 5 days.

Thus, as is the case with agar, the addition of protein or other organic substances has a distinctly deleterious effect: the virus

[11] The protein gelatin also is favored by its simplicity of composition. Its minimal molecular weight is estimated at 10,300 while that of hemoglobin at 50,000. For comparison, furthermore, the minimal molecular weight of egg albumen is stated as 33,800; of fibrin, 42,000; of serum globulin, 81,000; and of casein, 192,000 (see Cohn, E. J., Hendry, J. L., and Prentiss, A. M., *J. Biol. Chem.*, 1925, lxiii, 721).

requires for its survival only the simplest material. From this point, studies were therefore made on the effect of the gelatin alone.

We then employed a more thoroughly purified gelatin. Accordingly, we prepared the substance after the manner of Loeb for making isoelectric gelatin.[12] The method of preparation was as follows:

50 gm. of Poulenc powdered gelatin were added to 3 liters of M/128 acetic acid at 10°C. and stirred frequently. After 30 minutes the supernatant liquid was decanted, and fresh M/128 acetic acid at 10°C. (or in the cold room) was added to equal the original volume. The mass was frequently stirred and after ½ hour the acid was again decanted and replaced by an equal volume of distilled water at 5°C. The gelatin was well stirred and then filtered by suction in a Büchner funnel. It was then washed in the funnel 5 times with 5 liters of cold water. After all the water was drained off, the gelatin was removed and suspended in the buffer phosphate to a concentration of 10 per cent. It was then heated in the autoclave for sterilization on 3 successive days for 1 hour at 100°C. and the pH was adjusted to 7.6 between the heatings and after the final sterilization.

We then compared the effect of this buffered Loeb gelatin with buffered 0.25 per cent agar and found that the former permitted the survival of the virus for a much longer time than did the agar. For example in 1 experiment, the same sample of virus remained alive in the gelatin for 11 days at 34°C., but in the agar for less than 5 days. Hence the Loeb gelatin was not only superior to the same material washed by ordinary methods, but it also surpassed agar.

We then simplified the Loeb gelatin still further by omitting the buffer phosphate solution and adjusting the material to pH = 7.5 or 7.6 merely with potassium hydrate. Comparative tests revealed that the "adjusted" gelatin maintained the virus for longer periods than the buffered, at either 34°C. or 18–20°C., and under either aerobic or anaerobic conditions.

In the adjusted Loeb gelatin, the virus was kept alive for a longer period than in any of the other media tested. This period was longer than 69, but shorter than 100 days when the material was kept at room temperature (18–20°C.). In a parallel experiment, the active agent remained viable in 0.25 per cent agar for 45 days.

In this medium 3 successive subplants showed activity, as in the

[12] Loeb, J., Proteins and the theory of colloidal behavior, New York, 2nd edition, 1924, 41.

case with the agar. But we regard this fact as merely indicative of preservation of the virus rather than of its multiplication. For the 4th subplants—the critical ones, the ones in which the virus is diluted beyond its original limit of activity—were uniformly negative.[13]

At this point, when we had developed the optimum medium for the preservation of virus, namely, the adjusted Loeb gelatin, the limitation of time compelled us to discontinue the tests.

While these experiments were in progress we attempted the cultivation of the active agent in a variety of other special media and by different methods. We report these here for their general interest.

Miscellaneous Cultivation Tests.—Smith-Noguchi medium.[10] In this medium, the virus was incubated at 32–34°C., and subplants were made after 3 days. Material of the 1st subplant induced the typical experimental disease in all of 3 guinea pigs injected but that of the 2nd and of the 3rd successive transfers was inactive. In similar medium without the vaseline seal and incubated at 32°C. in an atmosphere of 10 per cent carbon dioxide combined with complete anaerobiosis identical results were obtained. In the Smith-Noguchi medium, therefore, the virus was preserved for about 3 days, or in the 1st subplant. But no evidence was obtained to indicate that the active agent could reproduce itself therein.

Tissue Cultures.—In tissue cultures made after the manner of Borrel, somewhat better results were obtained. These cultures were made with chicken plasma, chicken or guinea pig embryonic juice, and with fragments of embryonic epithelium. It was found possible to maintain the life of the virus through the 2nd subplant or for 4 days. The 3rd and subsequent successive transfers, however, were uniformly inactive. This method, therefore, also failed to favor multiplication of the virus. In the later transplants we frequently encountered secondary organisms so that the results of tissue cultures are inconclusive.

Adjusted Loeb 10 Per Cent Gelatin.—In this medium still better results were obtained. At 32–34°C. the virus was found in 3 successive subplants—in other words over a period of 9 days from the time of seeding. But later transfers were negative. Hence no multiplica-

[13] It is of interest that the 3rd subplants were maintained at a temperature of 34°C., as well as at 18–20°C. In either case the medium preserved the virus.

tion was noted here as well. Efforts were made to employ this medium in a 10 per cent carbon dioxide atmosphere but this method proved less favorable—the 1st subplant was the only one positive. The addition of a fragment of fresh rabbit kidney to this gelatin showed no better results, but this is additional proof that the requirements of the virus for life postulate the simplest structure of the medium. Whether our experiences with this medium can be interpreted to indicate that the conditions of preservation are distinct from those of multiplication or whether the medium can be still further improved to the point of making it a basic material for successful artificial cultivation, remains at present an open question.[14]

General Considerations on the Particulate and Living Character of the Virus.

We propose to consider the questions which have often been discussed as to whether the filter-passing virus of foot-and-mouth disease is an inanimate, chemical material, or a multiplying living body; or whether it is fluid, as postulated by the theory of *contagium vivum fluidum* (Beijerinck), or particulate. For a justification of experiments on artificial cultivation rests upon the assumption that the virus may be particulate and living.

With reference to the theory of *contagium vivum fluidum* it appears that the definition of this term is not in accord with the modern concept of the particulate structure of matter. The burden of proof that fluids, in general, may not be corpuscular rests then upon the

[14] We intend that this be the last paper of this series. The forthcoming report of the Commission to be published by the United States Bureau of Animal Industry should be consulted for additional miscellaneous studies by the writers and by Drs. Schoening and Traum. These include: Experiments on respiration of the virus, which were discontinued for want of a suitable control, and on intracutaneous reactions with concentrated virus. The latter tests revealed no practical procedure for skin diagnosis of foot-and-mouth disease, although it was noted that there was a certain cuticular sensitiveness of guinea pigs with the active experimental disease to the living virus. In addition it was found that earthworms could not be implicated as carriers and, contrary to the opinion of some observers, the virus is not fragile outside the animal body for it survived in earth for at least 25 days. In this report will also be found the observations of the Commission as a whole on epidemiology, immunity, and the clinic of foot-and-mouth disease, and some studies on the virus of vesicular stomatitis.

partisans of this theory. Apart from this we have presented what we believe as sufficient evidence that the active agent of foot-and-mouth disease is particulate. For by repeated and controlled filtration tests we have not only delimited measurement of individual active masses as relatively between 20 to 100 mμ, but also found that this relative size is constant.[15] The invariability of the limits of size contradicts, therefore, the notion that the incitant may be a "solute" varying in size in different "solvents." The electric charge carried by these particles, has, moreover, been determined by cataphoresis which can effect their separation from the protein substances in the medium containing virus.[15]

With regard to the question of the living character of the virus, its activity in extraordinarily high dilutions, its non-centrifugability, its remarkable resistance to such bactericidal agents as the narcotic solvents (alcohol, ether, chloroform) or such antiseptics as phenol, cresol, bichloride of mercury, etc., tend to support the idea that it is inanimate, or a chemical substance. We believe, nevertheless, that nothing has as yet been brought forward in these respects to prove conclusively that the active agent is inanimate. The factors just mentioned may represent reactions consequent upon its minuteness of size or its electropositive charge or both. It should be remembered that minute particles are subject to different physical laws than larger structures (Vlès[16]). For example, internal pressure, surface tension, and charge exert profound effects. However this may be, if each of the factors mentioned as opposed to the notion of a living nature of the incitant is analyzed, as will be done immediately, it will be noted that none is incompatible with this idea.

We have found that active materials are still infective in a dilution of 1:10,000,000.[1] This can be interpreted as merely indicating its minute size—yet sufficiently large in volume to contain from several hundred to several thousand protein molecules.[15] On the other hand, the action of the virus stops at a definite dilution and is directly proportional in respect to the length of incubation period and severity of symptoms upon its concentration. We have not encountered any effect similar to that noted in certain enzymatic actions, of nullifying antibodies in low dilutions.

[15] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 685.
[16] Vlès, F., *Rev. scient.*, 1921, lix, 294.

As for the inability to depose the virus in a fluid medium by centrifugation, we have already explained[1] that this method is not useful for sedimenting minute particles: whirling and convection currents and other forces hinder the deposition. This property is therefore also a consequence of its size and does not indicate, by itself, a fluid material.

The resistance to chemical reagents is, as we have shown, really illusory.[17] It is due to the fact that these reagents coagulate the proteins of the medium in which the virus is as a rule suspended. As a result the virus is protected by the coagulum and direct contact with the reagent becomes impossible. If coagulation is prevented, which can be done, the virus is then acted upon directly by the antiseptics. The incitant is then more rapidly destroyed by the chemicals than are living bodies such as staphylococci.

Finally, among analogies to living microorganisms, may be mentioned the epidemiological factors, the existence of at least 2 distinct types of virus, the more or less solid immunity which is induced, and a period of incubation which can at times last 12 days. Studies of these conditions will be described in detail in the Commission's complete report. On the other hand, if the virus is a living body, it appears to be of an order wholly different from that of known cultivable microorganisms. For, aside from its electropositive charge, deductive study of its requirements for artificial life indicates that the metabolism of the incitant is of a very simple kind and is quite limited to definite and inflexible conditions.

We may therefore conclude that nothing has as yet been presented to prove that the virus of foot-and-mouth disease is of a fluid character or inanimate nature.[18]

SUMMARY AND CONCLUSIONS.

No multiplication of the virus *in vitro* was observed. We have found, however, that the optimum conditions necessary for the preservation of the virus in artificial media are as follows:

[17] Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815.

[18] This problem is still being studied by one of the writers (O.) but with the virus of vesicular stomatitis of horses. This virus is similar in clinical manifestations and in many biological reactions, to be reported later, to those of foot-and-mouth disease. In a recent collaboration with Dr. F. L. Gates of The Rockefeller In-

The hydrogen ion concentration of the medium should be 7.5 to 7.6, not only at the beginning, but, and more important, at the conclusion of the period of observation. A strict anaerobic atmosphere is also favorable, as is a temperature below 37°C. A semisolid structure of the medium appears to be advantageous and this can be effected by the use of $\frac{1}{4}$th per cent agar or 10 per cent gelatin. Of the two, the gelatin is more desirable, and of the latter the most effective material is gelatin from which the impurities have been most thoroughly removed, namely the gelatin employed by Loeb for his isoelectric determinations. This material is best adjusted to the proper hydrogen ion concentration (7.5–7.6) with potassium hydroxide and not with buffer phosphate.

Gelatin is the simplest of protein media available and its employment is in keeping with the principle we have found that the requirements for life of the virus of foot-and-mouth disease are of the simplest. The addition of organic or complex protein substances, such as dextrose, broth, serum, lipoids, etc., to a simple basic medium interferes with the effectiveness of the latter. It is thus not surprising that we were unable to confirm the cultural results of Frosch and Dahmen for neither their medium nor its components or their method satisfies the essential conditions necessary to maintain artificially the life of the virus.

Furthermore, from the standpoint of technique it was found necessary when comparing 2 or more media for their value in preserving the incitant, to employ all of them in a parallel experiment with the same sample of virus, for the factors of potency of the active agent, contamination, and changes in hydrogen ion concentration, if variable, may give rise to faulty interpretations. Moreover, activity in 3 successive subplants may be regarded as mere preservation but not multiplication of the virus.

Finally, we discuss the status of the virus from the point of view of its fluid or particulate, and its animate or inanimate, characters.

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stitute it was found that the virus of vesicular stomatitis is destroyed by the same limits of energy and wave-length of ultra-violet light which can kill staphylococci. "Since the absorption of specific energies is one index of chemical character, and in this instance results in similar effects, these parallel reactions are indirect but suggestive evidence that the substance of the virus is similar in character and chemical constitution to bacterial protoplasm" (Olitsky, P. K., and Gates, F. L., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 431).

OXYGEN POISONING IN MAMMALS.

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Plates 27 and 28.

(Received for publication, December 20, 1927.)

INTRODUCTION.

The increasing use of oxygen in clinical medicine would seem to make worth while a study of the toxic effects which may result from its prolonged inhalation in high concentrations. The observation that oxygen inhalation may lead to pathological states is not a new one. And yet it has seemed important to us, because of our own constant use of oxygen inhalation as a therapeutic measure, to investigate this problem further, giving particular attention to the nature of the injury and the mechanism of death. The fact that many of the experiments done in the past were performed on animals housed in small containers with inadequate ventilation made it appear especially desirable to repeat experiments under conditions in which CO_2 removal, temperature, humidity and air motion were controlled, and to rule out definitely the possibility of death resulting from respiratory infections so prone to occur in caged animals and in rabbits in particular. It seemed not improbable that some of the pulmonary lesions described in the literature might be the result of respiratory infections perhaps resulting from a lowered resistance of lung tissue when exposed to an abnormal mixture of atmospheric gases.

Lavoisier is said to have remarked the poisonous effects of oxygen inhalation shortly after the discovery of this gas in the atmosphere. Regnault and Reiset (2) in 1849 demonstrated that the respiration of different species of animals in an atmosphere containing two or three times the normal concentration of oxygen remains unchanged. They found no alteration in the amount of oxygen consumed, in the respiratory quotient, or in the general behavior of the exposed animals. No further investigation in this field appears to have been made until the

classical experiments of Paul Bert (3) established the fact that oxygen at high tensions is a powerful poison. Bert showed that sparrows exposed to atmospheric air at 15 to 20 atmospheres pressure developed convulsions and died. If pure oxygen were used in the compression chamber only one-fifth as much pressure was needed to produce the same effects. On the other hand, if oxygen-poor air were used in the compression chamber the sparrows survived until overcome by the accumulated CO_2. Bert concluded that the toxicity of highly compressed air was due to the high tension of the contained oxygen, which he believed exerted a direct toxic effect upon the central nervous system. He was unable to reproduce the symptoms by the injection into a normal dog of a large amount of blood taken from a dog in oxygen convulsions. From this he argued that the status epilepticus which he described was not the result of a circulating poison secondarily produced by the oxygen. He found that high oxygen tensions had a similar effect on other laboratory animals and was also injurious to insects, arachnids, myriapods, molluscs, earthworms and germinating seeds. It inhibited the putrefaction of meat and delayed the souring of milk.

20 years later the problem was taken up by Lorrain Smith (4), who was the first to test out the effect of prolonged exposures to moderately high oxygen tensions. He found that mice suffered no ill effect from exposure for 8 days to 41.6 per cent of an atmosphere of oxygen, but that with 70 to 80 per cent of an atmosphere some mice died in 4 days of "congestion and consolidation" of the lungs, while others survived. At 1.14 to 1.50 atmospheres of oxygen all the mice died of consolidation of the lungs in 40 to 70 hours. At 1.66 to 1.89 atmospheres of oxygen mice, guinea pigs and larks died at 7 to 27 hours, the lung changes being similar. At 3.55 to 3.57 atmospheres of oxygen mice died in 5 hours. Mice which had begun to show dyspnea on exposure to this tension of oxygen died at once on being taken out of the chamber. Two larks exposed to 3.017 atmospheres of oxygen developed convulsions in 10 minutes. They were taken out of the chamber after 2 hours but both died. There was nothing noteworthy in their postmortem appearances. Lorrain Smith showed that by a previous short exposure to a high oxygen tension or by raising the oxygen tension very slowly the exposure could be carried distinctly beyond the point which usually produces convulsions. He also aimed to show that the toxic effect of oxygen is related to its tension in the inspired air and not its quantity in the blood. In a chamber containing 0.4 per cent carbon monoxide as well as 3 atmospheres of oxygen larks developed convulsions as usual, although the arterial blood at the end of the experiment was only 38 per cent saturated with oxygen.

In brief, Lorrain Smith demonstrated that moderately high tensions of oxygen produce an inflammation of the lungs, while very high tensions have an irritating effect on the central nervous system. He believed that the inflammatory reaction of the lung protected the central nervous system from pathological changes through interference with the diffusion of oxygen into the tissues. There is considerable variation between species and between individuals of the same

species in their susceptibility to oxygen. The minimum tension necessary to produce stimulation of the central nervous system is always well above that required to bring about pulmonary inflammation, but if the tension is sufficient the nervous symptoms develop rapidly and death may occur before there are any demonstrable changes in the lungs.

In 1903 Hill and McLeod (5) considered the question of the effect of compressed air on the respiratory exchange. From their experiments on mice they concluded, in agreement with an observation of Paul Bert, that a partial pressure of oxygen equal to 1 atmosphere does not increase, but rather lessens the processes of oxidation.

On the other hand, experiments in lower animal forms (6) have shown that the metabolism of many lower invertebrates, both marine and terrestrial, and some of the higher invertebrates such as the lobster and annelid worm is proportional over a wide range to the oxygen tension. This does not appear to be true of vertebrates.

Curiously enough, Lavoisier's original observation of the toxic effects of breathing high concentrations of oxygen at atmospheric pressure did not receive the attention of later workers (with the exception of Lorrain Smith) until Schmiedehausen (1) and David (7) confirmed his findings. They reported that pure oxygen supplied to a dog through a tracheal cannula produced a mild pulmonary hyperemia in as short a period as 15 minutes. 1 hour's exposure of a dog to 90 per cent oxygen in a chamber brought about definite hyperemia and extravasation of blood into the bronchi and alveoli. Similar results were noted in mice and guinea pigs, particularly after longer exposures. One guinea pig was exposed to 40 to 60 per cent oxygen at atmospheric pressure for 69 hours and 37 minutes. 6 hours after being taken out of the chamber into room air the animal died. Postmortem examination showed bronchopneumonia. This is the only case we have found recorded in the literature of pulmonary inflammation following exposure to increased oxygen tensions of less than 70 per cent of an atmosphere.

Benedict and Higgins (9), in 1911, carried out a series of experiments on the effect of inhalation of oxygen-rich mixtures for short periods of time on normal young men. With 40, 60 and 90 per cent oxygen they found no change in metabolism or respiration but a definite decrease in the pulse rate, which was more or less proportional to the percentage of oxygen breathed.

Bornstein and Stroink (10), in 1912, report the first case of experimental oxygen poisoning in man. Bornstein placed himself in a pressure chamber exposed to 2 atmospheres of pure oxygen. After 50 minutes he began to have cramps, first in the right, then in the left arm. The cramps ceased as soon as the pressure was lowered. These investigators also carried out a series of experiments on the effect of high oxygen tensions on dogs, apes, cats and rats. A dog and an ape, kept for several months in 0.6 atmosphere of oxygen, showed slight anemia,—an interesting observation in view of the opposite effect of low oxygen tensions.

Retzlaff (11), in 1913, found that the inhalation of oxygen produces vasoconstriction of the pulmonary blood vessels in the cat, and he suggests that the

beneficial effect of oxygen administration in cardiac failure with pulmonary edema may be ascribed to the improved pulmonary circulation thereby produced.

In 1916, Karsner (12) made an extensive study of the pathology of oxygen poisoning in rabbits, and recorded the time relationships of the various changes and gave a definition of the type of pneumonia occurring. He found that "80 to 96 per cent oxygen under normal barometric pressure produces in 24 hours, or more commonly 48 hours, congestion, edema, epithelial degeneration and desquamation, fibrin formation, and, finally, a pneumonia, probably of irritative origin," and described by him as a "fibrinous bronchopneumonia." He also found a certain degree of congestion in all of the abdominal organs which he believed to be secondary to the damage done to the pulmonary circulation. There were no demonstrable changes in the hematopoietic system other than congestion.

In a later work (13) Karsner and his coworkers showed that high partial pressures of oxygen are definitely inhibitory to the growth of certain strains of bacteria, while on other strains they may have no effect. The growth of pneumococcus was not inhibited by high oxygen tension.

Cleveland (14), in 1925, reported a series of ingenious experiments which indicated that oxygen in high concentrations is peculiarly toxic to intestinal protozoa. In cockroaches, for instance, the oxygen is 135 times as toxic for flagellates and 26 times as toxic for ciliates as for the host cockroaches. Similar effects are seen in termites and to a less marked degree in frogs, salamanders and goldfish, so that the intestinal tracts of these animals can be cleared of protozoa by simply exposing them to a sufficient oxygen tension. This test is impossible to carry out in warm blooded animals because the host is more susceptible than the parasite.

Barach (15), in 1926, published a series of carefully controlled experiments on the effect of oxygen-rich atmospheres on normal rabbits and on rabbits with pulmonary tuberculosis. He found that 60 per cent oxygen produced no effect on the general appearance, activity and weight or growth of normal rabbits over periods as long as 1 to 4 months. Furthermore, no gross or microscopic pathological change was observed for periods as long as 1 to 2 months. Attempts to increase the resistance of the pulmonary epithelium to atmospheres containing 80 to 85 per cent oxygen by previous exposure to lower concentrations were unsuccessful. In one case pulmonary edema followed the inhalation of 70 per cent oxygen for 12 days. On the basis of these observations Barach states that the highest concentration of oxygen compatible with safety for therapeutic use should be regarded as 60 per cent.

EXPERIMENTAL.

Our own studies on the toxic effects of oxygen began with observations on three dogs and three rabbits kept in an atmosphere of approximately 80 per cent oxygen. The animals were placed in cages in a large oxygen chamber used ordinarily for the treatment of pneumonia patients. In this chamber it was possible to maintain satisfac-

tory cleanliness and isolation of one animal from the other and to provide for the proper removal of carbon dioxide and moisture, and maintenance of fairly constant conditions of temperature, relative humidity and air motion. Daily observations of the weight, body temperature and behavior of the animals, and more frequent records of the room temperature, humidity and oxygen concentration were made. After a 4 day control period with the chamber open to the atmospheric air the doors were closed and oxygen was admitted into the chamber. The oxygen content of the chamber was raised at once to a little over 50 per cent of 1 atmosphere and then gradually, during the next 30 hours, to 80 per cent, where, with minor fluctuations, it was maintained throughout the remainder of the experimental period.

Experiments on Dogs.

The first abnormal sign noted in the dogs was the refusal of food. This was observed on the 3rd day after exposure to 70 to 80 per cent oxygen in Dog 1, a young, immature dog, on the 4th day in Dog 2, and on the 5th day in Dog 3. All of the dogs lost weight after the 3rd day. *Vomiting* occurred on the 5th day in Dog 1, on the 6th day in Dog 3 and was absent in Dog 2. *Drowsiness* was first recorded on the 6th day in all three dogs. *Labored breathing* was first noticed on the 6th day in Dog 3, on the 7th day in Dog 1 and was absent throughout in Dog 2. This respiratory distress became progressively worse during the rest of the experimental period. It had the character of slow, labored, deep breathing often associated with an apparent expiratory effort. There were no seemingly significant variations in the body temperature. Text-fig. 1 shows the variations in body weight and temperature in relation to O_2 and CO_2 content of the chamber.

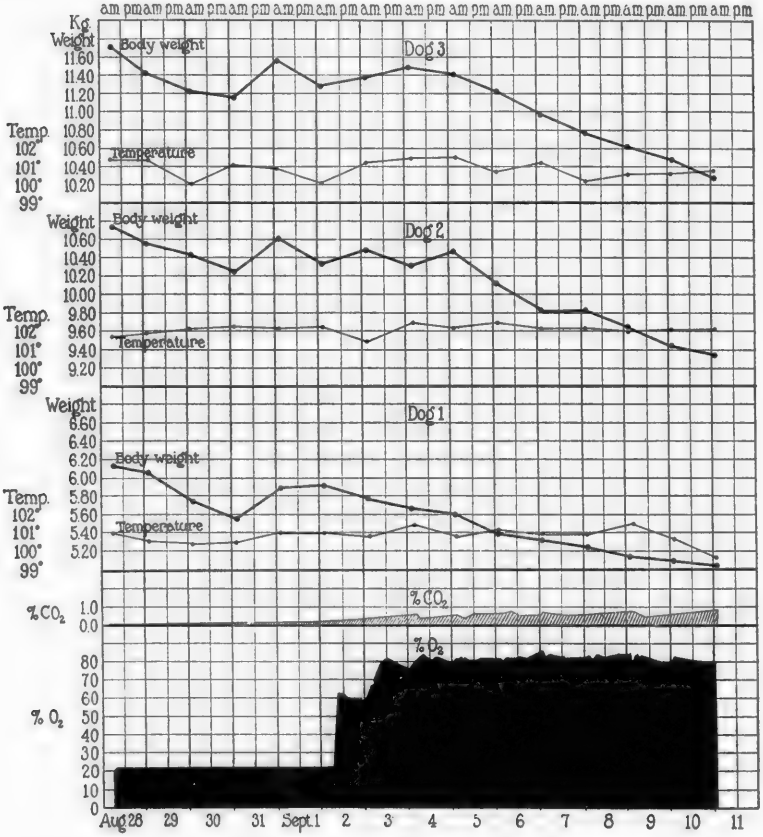
After 8 days exposure to 70 to 80 per cent of an atmosphere of oxygen the dogs were removed from the chamber and samples of their arterial blood were drawn for oxygen analysis by puncturing the femoral arteries.

Dog 1 was markedly cyanosed. There was a cardiac arhythmia, shown by electrocardiograph to be the result of extrasystoles. Blood-stained froth was pouring from the mouth. Breathing was labored. At each inspiration the flanks were sucked in and expirations were grunting. When turned on its back respiratory distress became very great. Analysis of the arterial blood showed an oxygen saturation of only 40 per cent.

Dog 2 showed no evidence of anoxemia or respiratory distress. The arterial blood was 94.6 per cent saturated with oxygen.

Dog 3, like Dog 1, though far less markedly, manifested respiratory disturbance. Respirations were slow and labored. The flanks sucked in with inspiration and expiration was accompanied by a prolonged groan. There was, however, no definite arterial anoxemia, the blood being 93.7 per cent saturated with oxygen.

The three dogs were then immediately and painlessly killed by the intravenous injection of from 10 to 20 cc. of a saturated solution of magnesium sulfate. This


am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm am pm
Kg
Weight
Body weight
Dog 3
Temp.
Temperature
Body weight
Dog 2
Temperature
Dog 1
Body weight
Temperature
% CO2
% O2
Aug 28 29 30 31 Sept. 1 2 3 4 5 6 7 8 9 10 11

TEXT-FIG. 1. Chart showing loss of weight of three dogs confined in chamber containing 80 per cent oxygen. The oxygen concentration is represented by the black area; the carbon dioxide by the narrow shaded area. Carbon dioxide concentration remained below 0.8 per cent throughout the experimental period.

method is admirable for the study of pulmonary pathology, as the heart beat and respirations are arrested simultaneously and at once, unaccompanied by an antemortem struggle.

Autopsies on Dogs.

The gross and microscopic appearance of the lungs in the three dogs showed changes in keeping with the severity of their response to oxygen. When examined by a pathologist* who was unfamiliar with the symptomatic course of these three animals the lesions were graded in order of intensity thus: Dog 1, Dog 3, Dog 2, which will be seen to correspond to the manifestations cited above.

Description of Lesions in Lungs of Dog 1.

When the thorax was opened the lungs were found to be collapsed. They were mottled, beefy red—bright in some areas and dull red in others. There were no normally pink lobes. The trachea was full of blood-tinged froth. There was no free fluid in the pleural cavities. The lungs were disturbed as little as possible, except for punctures made for the purpose of making cultures. They were fixed *in situ* by distending them with Helly's fluid injected through a tube tied in the trachea. The roots were then ligated and the lungs carefully removed from the thorax and placed in Helly's fluid. Histological preparations were made by the usual paraffin technique, sections being cut at 7 μ and stained with Giemsa and hematoxylin and eosin. The sections revealed a general destructive process of a non-infectious character involving all parts of both lungs, the chief features being: (1) capillary engorgement with some hemorrhage; (2) the presence of interstitial and intraalveolar serum; (3) hypertrophy and desquamation of alveolar cells; and (4) interstitial and intraalveolar infiltration of mononuclear cells.

No microorganisms were seen in any of the sections examined. It will be shown later that these lesions, though not so marked in the dog, are pronounced in the lungs of the rabbits exposed to oxygen in the same chamber with the dogs. A photograph of a characteristic section of lung tissue from Dog 1 is shown in Figs. 1 to 3.

Description of Lesions in Lungs of Dog 2.

On opening the chest wall the lungs were collapsed and of essentially normal appearance, being uniformly coral-pink. There was no evidence of infiltration or consolidation; nor was there any free fluid in the pleural cavities. The lungs were treated in the same manner as those of Dog 1. Microscopic examination showed little change except for an apparent capillary engorgement.

Description of Lesions in Lungs of Dog 3.

Both lungs were collapsed. The right lung was, for the most part, normally pink in color, but there were a few small hemorrhagic areas near the periphery, especially on the anterior borders of the ventral lobe. The left lung had several small hemorrhagic areas on the surfaces of all lobes which were otherwise of normal

* Dr. L. T. Webster of the Staff of The Rockefeller Institute, whom we wish to thank for his help in interpreting the nature of the lesions.

appearance. There was no free fluid in the pleural cavities. Microscopic examination showed capillary engorgement with some hemorrhage. Fig. 4 shows a photomicrograph of a characteristic region taken from the right lower lobe of Dog 3.

The gross appearance of all the other viscera was essentially normal.

Attempts at Cultivation of Bacteria.

Broth cultures taken from the heart's blood in all three dogs were sterile, and material from the lung punctures streaked on blood agar also showed no bacterial growth.

Experiments on Rabbits.

Three rabbits, housed in separate cages, were placed in the same chamber with the dogs just described, and exposed to identical atmospheric conditions as the dogs. Daily observations of body temperature and weight were made as well as careful inspection of behavior. No significant fluctuation in temperature occurred. All of the rabbits showed a slight loss of weight during the experimental period. *The first symptom of significance was marked dilatation of the alæ nasi accompanying respiration.* This was noticed on the 6th day after exposure to 70 to 80 per cent oxygen in Rabbit 1; on the 6th day in Rabbit 2; on the 5th day in Rabbit 3. From their general appearance at this time the prediction was made that they would die in the order in which they eventually did die, namely No. 3 first, then No. 1 and finally No. 2. The dilatation of alæ nasi was followed by definite signs of respiratory distress, gasping inspirations with the use of the accessory muscles of respirations, the mouth opening wide with each inspiration. This was accompanied by definite cyanosis of the tissues about the nose and of the ear tips. These symptoms appeared from the 6th to 7th day in all three animals. The intensity of the respiratory distress grew worse, the animals showing marked orthopnea when turned on their backs for the purpose of taking their temperatures. Rabbits 1 and 3 died on the 7th day after exposure to 70 to 80 per cent oxygen.

From the appearance of cyanosis and the low oxygen saturation found in the arterial blood of Dog 1 it was believed that the immediate cause of death was probably anoxemia resulting from the lesion of the respiratory membrane. To test this point further it was decided to take Rabbit 2 out of the chamber, with the anticipation of its being immediately asphyxiated by the sudden lowering of alveolar oxygen tension. When this was done the animal at once had a convulsive seizure and died in a few moments after exposure to atmospheric air.

Autopsies on Rabbits.

With minor variations in intensity of reaction the gross and microscopic appearance of the three rabbits' lungs were closely similar. All three showed mottled dull to beefy red discoloration—with much gross edema, and either froth or fluid in the trachea. Rabbit 1 had 2 to 3 cc. of blood-tinged fluid in both pleural cavi-

ties, which, on direct smear, showed many lymphocytes and leucocytes and fibrin, but no bacteria. A few cc. of a similar but not blood-tinged fluid was found in the pleural cavities of Rabbit 2. None was seen in Rabbit 3.

Microscopic examination of the lungs of the three rabbits showed in varying degrees of severity the same reaction as seen in Dog 1, though on the whole a more intense one, *viz.:* capillary engorgement with hemorrhage, interstitial and intra-alveolar serum, hypertrophy and desquamation of alveolar cells, interstitial and intraalveolar infiltration of mononuclear and of a few eosinophilic cells. No microorganisms were found in any of the sections examined. Photomicrographs of sections from the lungs of Rabbits 1 and 3 are shown in Figs. 5 and 6. Heart's blood cultures were sterile. With the exception of an occasional mold and colony of large spore-bearing bacillus most of the plates streaked with material obtained from lung puncture were likewise sterile.

Experiment on a Guinea Pig.

Protocol.—December 3. A guinea pig was placed under a bell jar, so arranged on a wooden platform that oxygen could be blown into the jar at the desired rate. A basket containing soda-lime, for the removal of CO_2, was suspended in the jar. At 4.25 p.m. the flow of oxygen through the jar was begun at the rate of 1 liter a minute.

December 4. The guinea pig looks normal. There is no dyspnea. Analysis of sample of gas in the bell jar: Oxygen, 99 per cent. Carbon dioxide, 0.67 per cent.

December 6. 12 noon. Respirations deep.

5 p.m. No apparent dyspnea. The guinea pig has eaten lettuce and some oats.

December 7. 9 a.m. Found dead. Autopsy showed the lungs to be a deep, dull red, resembling liver in appearance. Sections of the lungs placed in 10 per cent formalin sank to the bottom of the container.

Experiments on Mice.

Four full grown mice were placed by pairs into two dialyzing jars which were found to be convenient receptacles for subjecting them to the desired gas mixtures. The jars were partly filled with wood shavings to serve as bedding for the mice and provide for their warmth. Two of the mice acted as controls and two of them were to be subjected to a high concentration of oxygen. Compressed air was run into the control jar from a pressure tank at a rate of 1 liter per minute and oxygen into the other. This rate of flow provided for an adequate removal of CO_2 and moisture. It was believed that any harmful influence which might result from the effect of compression and commercial handling of oxygen might be controlled by the use of air compressed to the same degree in cylinders identical to those containing the oxygen.

Protocol.

Control Mice.

Mouse marked with mercurochrome. Weight 25 gm.

Unmarked mouse. Weight 19 gm.

Nov. 16, '25, 4.30 p.m. Air flow started at 1 liter per min.

Nov. 17, '25. Mice active. Buried in shavings most of the time. No condensation of moisture on walls of jar.
Analysis of air in jar: O_2 = 22.09 per cent.
CO_2 = 0.22 per cent.

Nov. 18, '25. Mice appear normal.
Analysis of air in jar: O_2 = 21.58 per cent.
CO_2 = 0.25 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25. Mice appear normal.
Analysis of air in jar: O_2 = 21.68 per cent.
CO_2 = 0.4 per cent.
4.30 p.m. Compressed air tank nearly empty. Jar connected with house air pressure system and air run in at the same rate of flow, *viz.:* 1 liter per min.

Oxygen Mice.

Mouse marked with picric acid. Weight 19.6 gm.

Mouse marked with carbolfuchsin. Weight 20 gm.

Nov. 16, '25, 4.30 p.m. O_2 flow started at 1 liter per min.

Nov. 17, '25. Mice appear normal.
O_2 concentration in jar 98 per cent.

Nov. 19, '25. Mice appear normal. They are lively and restless. They remain on top of the shavings, whereas control mice are buried.
O_2 concentration in jar 98 per cent. CO_2 0.39 per cent.

Nov. 20, '25. Mice appear normal.
O_2 concentration 98.5 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25, 11.30 a.m. Mouse marked with picric acid is found dead. Weight 15.8 gm. Mouse marked with carbolfuchsin is gasping for breath. Its mouth opens wide and its whole body shakes at each inspiration. Cyanosis of tail, feet and nose. 2.45 p.m. Mouse dead. Weight 15.3 gm.

Nov. 23, '25. Mice appear normal.

Nov. 24, '25. Mice appear normal.

Nov. 25, '25. Mice appear normal.
Mouse marked with mercurochrome now weighs 27.6 gm. Unmarked mouse now weighs 20.6 gm. Jar cleaned and fresh wood shavings put in. Flow from compressed air cylinder again begun at rate of 1 liter per min.

Nov. 27, '25. Mice appear normal.

Nov. 28, '25, 8.40 p.m. Mice appear normal.

Nov. 29, '25, 10.30 a.m. Mice appear normal.

Nov. 30, '25. Mice removed from jar. They are active and normal in appearance.
Mouse marked with mercurochrome weighs 27.1 gm.
Unmarked mouse weighs 20.5 gm.

Autopsy of both these mice showed the lungs to be uniformly discolored a deep red, almost indistinguishable from liver. The lungs sank when placed in Zenker's fluid.

The experiments thus far reported demonstrate quite clearly, we believe, that for several mammalian species, the mouse, guinea pig, rabbit and dog, inhalation of commercial oxygen in concentrations greater than 70 per cent of an atmosphere may lead to a train of physiological changes consisting of drowsiness, loss of appetite, loss of weight, dyspnea and cyanosis, usually culminating in death from extreme oxygen want. The cause of the oxygen want is undoubtedly to be sought for in the acute pulmonary changes, to be characterized as a diffuse hemorrhagic edema of the lungs found in all these species.

Is it possible that this destructive process is due not to the oxygen itself but to some impurity in it introduced in the process of manufacture? The oxygen used thus far in this work was commercially prepared by a method now commonly employed, namely: the fractional distillation of liquid air. Before concluding that the pathological effect is due to oxygen and not to the presence of impurities, parallel experiments were done on mice kept in jars containing oxygen prepared in two differing ways: (1) by the so called "air reduction" process, (2) by the electrolytic dissociation of water.

This experiment shows that mice confined in a jar containing 96 to 98 per cent oxygen electrolytically prepared show the same physiological and pathological reactions as mice confined in equivalent concentrations of oxygen prepared by the "air reduction" process.

Burrows (16) reported the observation that oxygen was inhibitory to the growth of tissue cultures only when the gas had been led through rubber tubing, and believed that the toxic effect was due to a substance formed by the reaction of oxygen or ozone with rubber. Burrows, however, prepared his oxygen by the electrolysis of dilute H_2SO_4. By this method there is usually a contamination with ozone, which Burrows removed by bubbling the gas through olive oil. Under these circumstances it was not inhibitory to the growth of his culture material.

To control this factor two mice were placed in a dialysis jar connected with a tank of electrolytically prepared oxygen. The oxygen from the cylinder was led through glass tubes and bubbled successively through three wash bottles containing respectively 2M H_2SO_4, 2M NaOH and an approximately 10 per cent solution of K_2MnO_4. 4 days after the flow of oxygen was begun both mice were gasping for breath and incoordinated in their motions. The following day one of

Protocol.

Air Reduction Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked.
3.50 p.m. O_2 flow started at 1 liter per min.

Nov. 25, '25, 2.20 p.m. Both mice appear normal.
O_2 concentration 96 per cent.
CO_2 concentration 0.27 per cent.

Nov. 27, '25, 11.50 a.m. Mice appear drowsy and inactive.
O_2 concentration 99 per cent.
CO_2 concentration 0.41 per cent.

Nov. 28, '25, 8.40 p.m. Mice alive but very dyspneic.

Nov. 29, '25, 10.30 a.m. Safranine-marked mouse is dead. Second mouse living but very dyspneic. Moves about actively, however. Coat is ruffled.
O_2 concentration 97 per cent.
CO_2 concentration 0.11 per cent.

Nov. 30, '25. The surviving mouse breathing very rapidly, but not gasping.
O_2 concentration 98 per cent.

Dec. 2, '25. Died at 11 a.m. Autopsy shows usual picture of red liver-like appearance of lungs. The removed lungs sink when placed in distilled water.

Electrolytic Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked.
3.50 p.m. O_2 flow started at 1 liter per min.

Nov. 25, '25, 12.30 p.m. Mice lively. Appear normal.
O_2 concentration 96 per cent.
CO_2 concentration 0.55 per cent.

Nov. 27, '25, 11.30 a.m. Mice appear normal, but drowsy.
O_2 concentration 98 per cent.
CO_2 concentration 0.50 per cent.

Nov. 28, '25, 8.40 p.m. Both mice very dyspneic.

Nov. 29, '25, 10.30 a.m. Severe dyspnea.
4.30 p.m. Unmarked mouse gasping. Lower jaw drops with each inspiration. Other mouse looks sick but is not gasping. Both move about when jar is shaken. Coats are ruffled.
O_2 concentration 96 per cent.
CO_2 concentration 0.18 per cent.

Nov. 30, '25. Both mice gasping. Noses and tails dusky bluish. Have eaten very little.

Dec. 1, '25. Both mice found dead. Lungs are deep purple-red the color of liver. Sink in water.

them was dead and the other, when moribund, was etherized. The lungs of both showed the usual changes.

In another experiment oxygen was bubbled through olive oil for the purpose of removing traces of ozone, even though its presence could not be shown by the starch-iodine test on the gas as it emerged from the cylinder. Mice exposed to this oxygen died of the same symptoms and showed the same autopsy findings as all the others described above.

DISCUSSION.

The phenomena described in this paper seem to have general application. The mammalian organism can survive exposure to oxygen concentrations varying roughly from 6 per cent to 60 per cent of an atmosphere. Beyond these limits it rapidly deteriorates, and at each extreme, death results from oxygen deprivation. At the lower limit oxygen want results from the diminished alveolar oxygen tension. At the upper limit, although alveolar oxygen tension is far above normal, the diffusion membrane of the lung is so damaged that in spite of the increased head of pressure in the alveoli, the arterial blood remains unsaturated, and the animal dies from anoxemia. This process possibly is of a "protective reaction" nature on the part of the organism, but going too far culminates in death. The organism, in its unsuccessful effort to achieve a new equilibrium, is kept alive by the very environmental condition which ultimately destroys it.

SUMMARY AND CONCLUSIONS.

1. Oxygen in concentrations of over 70 per cent of an atmosphere is poisonous to dogs, rabbits, guinea pigs and mice.

2. The poisonous effects manifest themselves in drowsiness, anorexia, loss of weight, increasing dyspnea, cyanosis and death from oxygen want.

3. The cause of oxygen want is a destructive lesion of the lungs.

4. The lesion may be characterized grossly as an hemorrhagic edema. Microscopically there is to be seen in varying degrees of intensity (*a*) capillary engorgement with hemorrhage, (*b*) the presence of interstitial and intraalveolar serum, (*c*) hypertrophy and desquama-

tion of alveolar cells, (*d*) interstitial and alveolar infiltration of mononuclear cells.

5. The type of tissue reaction is not characteristic of an infectious process and no organisms have been recovered at autopsy from the heart's blood or from lung puncture.

6. The poisonous effects of inhalations of oxygen-rich mixtures do not appear to be related to impurities in the oxygen, nor are they related to faulty ventilation, excessive moisture or increased carbon dioxide in the atmosphere of the chambers in which the experimental animals were confined.

BIBLIOGRAPHY.

1. Schmeidehausen, P. G., Die pathologisch-anatomischen Veränderungen der Lungen bei verändertem Sauerstoffgehalt der Atemluft, Inaugural dissertation, Halle, 1909.
2. Regnault, V., and Reiset, J., Versuche über das Athmen der Thiere, *Ann. chim.*, 1849, xxvii, 299.
3. Bert, Paul, La pression barométrique, Paris, 1878.
4. Smith, J. Lorrain, The pathological effects due to increase of oxygen tension in the air breathed, *J. Physiol.*, 1899, xxiv, 19.
5. Hill, L., and MacLeod, J. J. R., The influence of compressed air on the respiratory exchange, *J. Physiol.*, 1903, xxix, 492.
6. Amberson, W. R., Mayerson, H. S., and Scott, W. J., The influence of oxygen tension upon metabolic rate in invertebrates, *J. Gen. Physiol.*, 1924–25, vii, 171.
7. David, O., Versuche über den Einfluss sauerstoffarmer Luft auf künstlich geschädigte Lungen, *Z. exp. Path. u. Therap.*, 1912, xi, 239.
8. David, O., Versuche zur Erzeugung von Lungenhyperämie, *Z. klin. Med.*, 1912, lxxiv, 404.
9. Benedict, F. G., and Higgins, H. L., Effects on men at rest of breathing oxygen-rich gas mixtures, *Am. J. Physiol.*, 1911, xxviii, 1.
10. Bornstein, A., and Stroink, Ueber Sauerstoffvergiftung, *Deutsch. med. Woch.*, 1912, xxxviii, 1495.
11. Retzlaff, K., Der Einfluss des Sauerstoffs auf die Blutcirculation in der Lunge, *Z. exp. Path. u. Therap.*, 1913, xiv, 391.
12. Karsner, H. T., The pathological effects of atmospheres rich in oxygen, *J. Exp. Med.*, 1916, xxiii, 149.
13. Karsner, H. T., Brittingham, H. H., and Richardson, M. L., Influence of high partial pressures of oxygen upon bacterial cultures, *J. Med. Research*, 1923–24, xliv, 83.
14. Cleveland, L. R., Toxicity of oxygen for protozoa *in vivo* and *in vitro:* animals defaunated without injury, *Biol. Bull.*, 1925, xlviii, 455.

15. Barach, A. L., The effects of atmospheres rich in oxygen on normal rabbits and on rabbits with pulmonary tuberculosis, *Am. Rev. Tuberc.*, 1926, xiii, 293.

16. Burrows, M. T., The oxygen pressure necessary for tissue activity, *Am. J. Physiol.*, 1917, xliii, 13.

EXPLANATION OF PLATES.

PLATE 27.

FIGS. 1 and 2. Photomicrographs of sections of right lower lobe of lung of Dog 1. Magnification × 130. The pictures show the intraalveolar transudation of serum and red blood corpuscles.

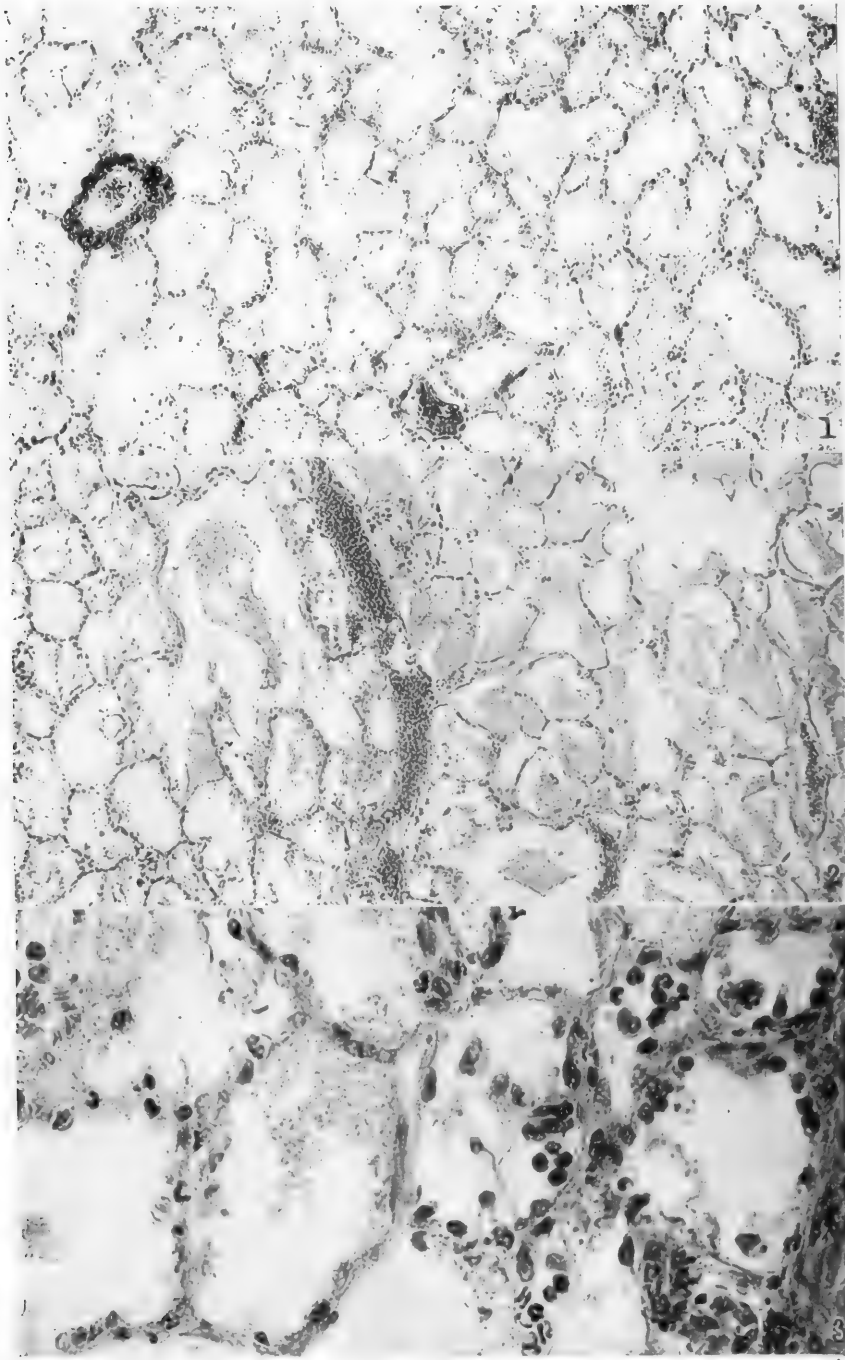
FIG. 3. Section of right lower lobe of Dog 1. Magnification × 500. The picture shows desquamated alveolar epithelial cells.

PLATE 28.

FIG. 4. Section of lower lobe of right lung of Dog 3, showing engorged and tortuous alveolar capillaries. Magnification × 130. This probably represents the initial hyperemic stage before hemorrhage and edema have occurred.

FIG. 5. Section of lung of Rabbit 1. Magnification × 130. The picture shows transudation of serum and cells as well as cellular exudate.

FIG. 6. Section of lung of Rabbit 3. Magnification × 500. The picture shows desquamation of alveolar epithelial cells as well as an exudate consisting chiefly of mononuclear and a few eosinophilic cells.



(Binger, Faulkner, and Moore: Oxygen poisoning in mammals.)

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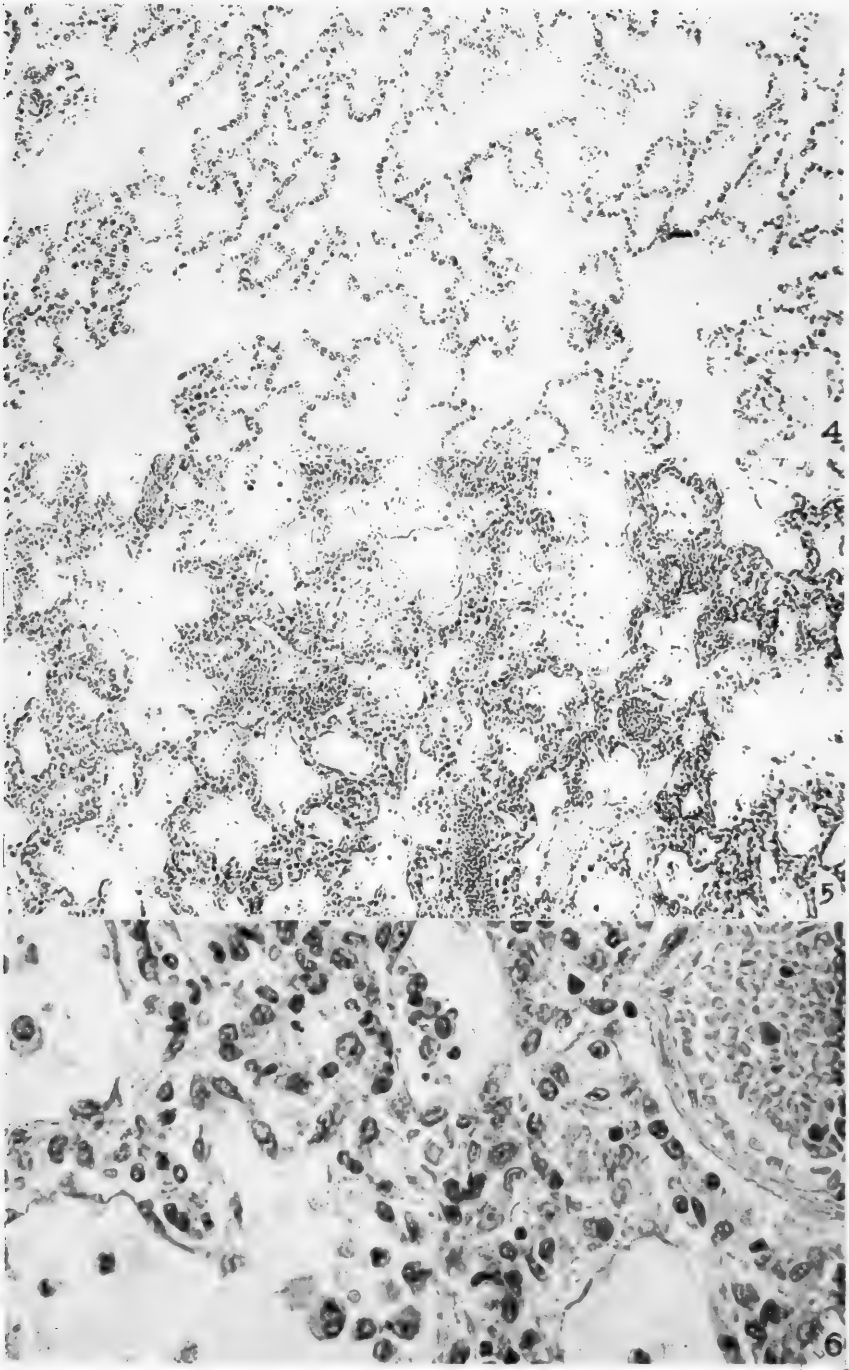

(Binger, Faulkner, and Moore: Oxygen poisoning in mamm[illegible])

OXYGEN POISONING IN COLD BLOODED ANIMALS.

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PLATE 29.

(Received for publication, January 3, 1927.)

INTRODUCTION.

In another paper (1) the effect of breathing atmospheres rich in oxygen on a variety of mammals was reported, and the nature of the injury to the lungs and the cause of death was discussed. It was pointed out that the pulmonary lesion was characterized by capillary dilatation and hemorrhagic edema. The work here to be described was undertaken not simply for the purpose of extending the study to other species but with the hope of observing the effect of high concentrations of oxygen on a capillary system more accessible to inspection than that in the lungs. For this purpose we utilized the web of frogs exposed to high concentrations of oxygen (90 to 99 per cent) over long periods. The web capillaries were examined periodically during life and after death sections were made of the skin, as well as of the lungs, heart, liver and kidney. No changes were observed in the capillaries or in the pigment cells as a result of oxygen administration. Two frogs grew definitely darker in color after 7 and 10 weeks exposure, respectively, but we attach no special significance to this, because color changes in the frog's skin are readily produced by a variety of causes difficult to control (2). In this instance inanition may have been a cause, since the frogs were not fed during the course of the experiment.

Krogh (3) has shown that the cutaneous respiratory exchange of the frog differs from its pulmonary respiration in that carbon dioxide is eliminated chiefly through the skin, while oxygen is absorbed chiefly by the lungs. Enough oxygen may be absorbed through the skin, however, to keep a frog alive for several weeks at 7-8°C. when the pulmonary ventilation is entirely cut off (4). This may be

accomplished by applying a gag to the frog in such a manner that it is unable to close its mouth, thus inhibiting the normal swallowing movements, whereby this animal forces air into its lungs (5). By placing gagged frogs in jars containing 90 to 95 per cent oxygen it was hoped to bring about changes in the skin capillaries, since the oxygen absorption would, under these circumstances, be chiefly through the skin and pharyngeal mucous membrane. None of the frogs showed any changes, however, in the skin capillaries.

These experiments, though negative with respect to the original subject of enquiry, brought out the fact that frogs could survive, apparently unharmed, in concentrations of oxygen poisonous to mammals. This observation seemed of sufficient interest to merit further study. Did it signify that cold blooded animals are resistant to the poisonous action of high concentrations of oxygen? And is the difference in their behavior from the behavior of mammals solely dependent on their differences in body temperature?

EXPERIMENTAL.

Experiments on Frogs.—Two frogs placed in a shallow dish containing a little water were continuously exposed under a bell jar to an atmosphere with an oxygen concentration averaging from 80 to 90 per cent. After 21 days exposure they were removed from the jar. Throughout the experiment and at the time of removal from the oxygen they remained apparently normal in appearance and behavior. The frogs were killed and autopsied. No gross changes in the lungs or other viscera were to be seen. Two other frogs were kept continuously in 90 to 98 per cent oxygen for respective periods of 49 and 68 days without apparent damage to their well being. At the close of the experiment they were found to be more deeply pigmented than they had been at the start, forming a marked contrast in color to two control frogs. No other changes in them were to be seen. Mammals exposed to similar environmental conditions would probably have died within the 1st week. To discover whether this difference in behavior was due to differences in body temperature an effort was made to keep frogs alive at incubator temperature (37.5°C.), but the frogs were unable to withstand this and died within 24 hours. For this reason we decided to make experiments on turtles whose life habits frequently expose them to high temperatures.

Experiments on Turtles.—Is the turtle also resistant to high concentrations of oxygen?

Protocol. Experiment 1.—A common adult pond turtle (*Chelopus guttatus*) was placed in a shallow dish containing a little water. The dish was covered with a

bell jar through which a stream of oxygen was allowed to run at the rate of 1 liter per minute. After 23 days exposure to an atmosphere containing an oxygen concentration for the most part above 90 per cent the turtle was removed in an apparently normal state. At autopsy the lungs were found to be normal in both gross and microscopic appearance (see Fig. 1).

Experiment 2.—The above experiment was repeated on another adult of the same species. After 10 days in an atmosphere of 90 per cent oxygen the turtle was removed from the bell jar in an apparently normal state.

Experiment 3.—Five young green turtles (*Chrysemes elegans*) lived for 3 weeks in 97 to 99 per cent oxygen at room temperature (23–26°C.) without appearing in any way affected by the unusual environment. A sixth member of this group was found dead after 17 days exposure, but there was no respiratory distress observed, and no definite evidence of pulmonary pathology was seen. These experiments indicate that the turtle, like the frog, can survive, unharmed, in concentrations of oxygen fatal to mammals.

Effect of Mammalian Temperature on Turtles.

Two adult specimens of *Chelopus guttatus* survived for 36 days in an incubator at 37.5°C., showing at no time loss of appetite or other signs of ill health.

An adult *Chrysemes elegans* spent 34 days, and six young individuals of the same species spent 21 days in the incubator. At the end of this time they were all lively and eating well.

From these observations it can be concluded that turtles are unharmed by prolonged exposure to room air warmed to 37.5°C.

Effect of Combination of High Concentrations of Oxygen and Mammalian Temperature on Turtles.

To find out whether the difference in the reaction of turtles exposed to high concentrations of oxygen from that of mammals was dependent upon their differences in body temperature, we exposed turtles to 90 per cent oxygen warmed to 37.5°C. Individuals of various ages and species were confined in an ordinary bacteriological incubator in which the oxygen concentration was kept above 90 per cent by blowing a stream of oxygen through it. It was soon learned that under these conditions turtles behaved like mammals in their susceptibility to oxygen, exhibiting like abnormalities in function and structure. Since young turtles behaved somewhat differently from adults the experimental results will be classed accordingly.

Experiments on Adult Turtles.

Protocol. Experiment 4.—May 22, 1926. An adult male pond turtle (*Chelopus guttatus*), kept in a shallow dish containing a little water, was put into an ordinary bacteriological incubator. This individual had previously survived, without ill effect, a 36 day exposure to room air warmed to 37.5°C. The incubator was provided with a pan of soda-lime for absorption of carbon dioxide, and a constant stream of oxygen was now run through the incubator at the rate of 1 liter per minute. Analysis at 11 p.m. showed the oxygen percentage to be 94 and the temperature 37.5°C.

May 23, 11 a.m. Oxygen concentration in the incubator 91 per cent.

May 24, 9.45 a.m. Oxygen concentration in the incubator 94 per cent. CO_2 concentration in the incubator 0.02 per cent.

At about 11 a.m. it was noticed that the turtle was opening its mouth to breathe. It stretched its neck, opened its mouth and extended its fore legs at each inspiration. These gasping breaths increased in frequency during the afternoon.

May 25. At 10 a.m. the turtle was found dead. Autopsy showed the lungs to be opaque and blood-red instead of being of the normal pale gray transparency. Microscopic examination showed marked engorgement of the pulmonary capillaries.

Experiment 5.—May 5, 1926. Another adult turtle of the same species (*Chelopus guttatus*) was placed in an incubator containing 90 per cent oxygen, as in the experiment just reported.

May 6, 9.10 a.m. O_2 concentration 93 per cent.

7.45 p.m. O_2 concentration 94 per cent. Temperature in incubator 37.5°C. Turtle rather inactive and refuses to eat an earthworm which is offered to it.

May 7, 12.30 p.m. O_2 concentration 95 per cent. Temperature 37°C.

May 8, 11 a.m. O_2 concentration 97 per cent. Temperature 38°C.

May 9, 12.30 p.m. O_2 concentration 95 per cent. Temperature 38.5°C.

May 10, 10 a.m. O_2 concentration 95 per cent. Temperature 38.5°C. The turtle shows a peculiar form of breathing today. It stretches its neck, then opens its mouth for several seconds and swells out its throat. It then shuts its mouth, retracting and deflating its neck, apparently forcing air into the lungs. This process is repeated about every 30 seconds.

May 11, 10.30 a.m. O_2 concentration 95 per cent. Temperature 38°C. Turtle still shows same type of breathing at a somewhat more rapid rate than yesterday. Accompanying each inspiratory gasp it extends its fore legs, as if to aid respiration.

May 12, 9.40 a.m. O_2 concentration 94.5 per cent. Temperature 37°C. Same type of breathing persists.

May 13, 9.30 a.m. Oxygen cylinder emptied during night. O_2 concentration fell to 42 per cent. Gasping for breath.

10 a.m. O_2 concentration 97 per cent.

1.15 p.m. Turtle apparently lifeless—lying with its head under water. Reflexes were still present, however. The plastron was removed and the heart was

found to be still beating. All the organs except the lungs were removed. These were left *in situ* and fixed in 10 per cent formalin. The lungs were definitely redder than normal. Microscopic examination showed a very marked vascular congestion with great dilatation of capillaries. An illustration of this may be seen in Fig. 2, which should be compared with Fig. 1, a section made from the normal turtle lung.

Experiments similar to the two just described were repeated on another individual of the same species, on two adult Cumberland terrapins (*Chrysemes elegans*) and on one box turtle (*Cistudo carolina*) approximately 15 years old. All of them ended fatally. The duration of life and the degree of pulmonary congestion found at autopsy varied in the different individuals.

These experiments show that high concentrations of oxygen, when combined with incubator temperature (37-38°C.), are fatal to adult turtles of several different species, producing changes analogous to those found in the mammal, namely: progressive dyspnea, culminating in death, with hemorrhagic changes in the lungs. Neither high concentrations of oxygen, nor warm room air alone brought about these changes.

Experiments on Young Turtles (Chrysemes elegans).

It has already been stated that young individuals of the species *Chrysemes elegans* survived unharmed exposure to 97 to 99 per cent oxygen for a prolonged period. They were found to be equally resistant to exposure to warm air, behaving in these respects just like the adults.

On exposing them to oxygen warmed to 37.5°C., it was observed that they survived longer than the adult specimens did. Eventually, however, they succumbed, showing similar hemorrhagic extravasations into the lungs.

Experiment 6.—Five young turtles (*Chrysemes elegans*), whose shells measured about 3.5 cm. in diameter, were exposed in an incubator at 37.5°C. to an oxygen concentration varying from 94 to 98 per cent of an atmosphere. After 21 days exposure, the first one died. After 24 days, a second died. After 27 days, two of the survivors showed curious wing-shaped dark areas on their backs, found at autopsy to be due to seepage of extravasated blood from the lungs. These two died respectively on the 32nd and 41st day after exposure. The lungs at autopsy were found to be very red, with extravasation of blood into the extrapulmonary tissue.

The last turtle died on the 50th day after exposure to warm oxygen. At autopsy its lungs were found to be red and edematous, with blood stains through the plastron and carapace.

DISCUSSION.

An adequate explanation for the facts presented in this paper is not at hand. It is clear that increasing the temperature does not increase the oxygen concentration in the body. The implication would seem to be that a reaction occurs between oxygen and pulmonary tissue whose temperature coefficient is such that it progresses at the temperature of the mammalian body but not at ordinary room temperature. Whether a chemical substance is produced which acts as an irritant to the lungs cannot be stated, or whether the increased temperature acts by raising the metabolic rate, which is known to enhance the toxic effect of certain substances.

SUMMARY AND CONCLUSIONS.

1. Exposure of frogs to atmospheres containing approximately 95 per cent of oxygen is without apparent effect on their state of well being, and produces no noticeable changes in the appearance of their web capillaries.

2. Turtles exposed to similar atmospheres are also apparently unaffected unless the oxygen be warmed to mammalian temperature.

3. At this temperature (37.5°C.) the turtles behave like mammals, showing loss of appetite, shortness of breath, death and, at autopsy, hemorrhagic extravasations in the lungs.

4. Young turtles are more resistant (or adaptable) to this change in environment than mature ones.

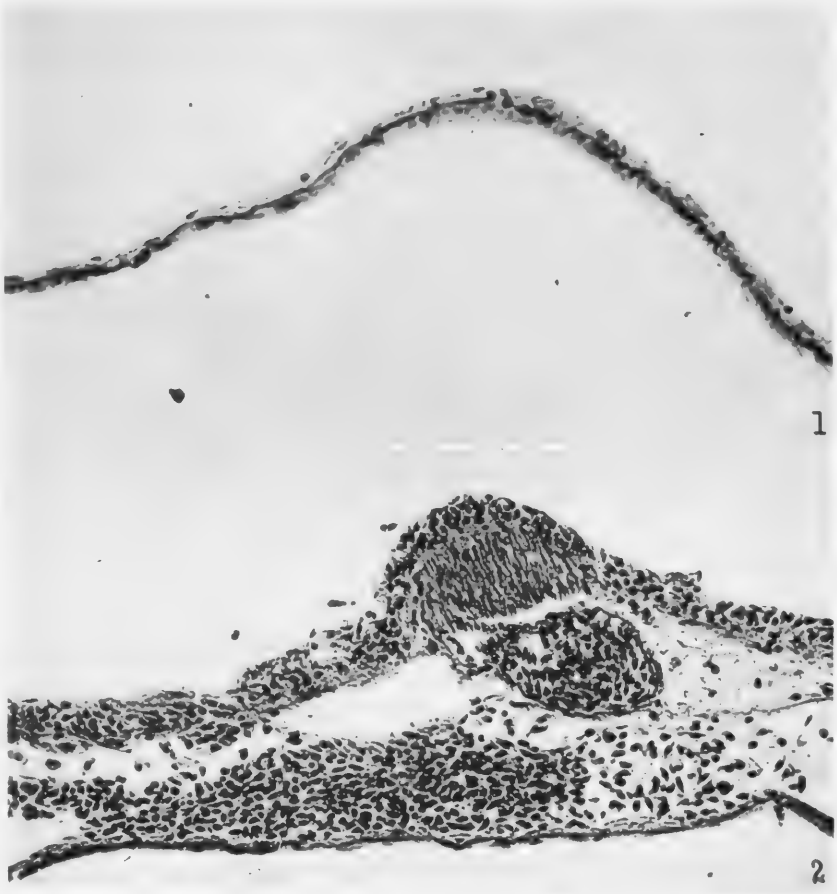
BIBLIOGRAPHY.

1. Binger, C. A. L., Faulkner, J. M., and Moore, R. L., *J. Exp. Med.*, 1927, xlv, 849.
2. Gaupp, E., in Ecker, A., and Wiedersheim, R., Anatomie des Frosches, Braunschweig, 2nd edition, 1904, pt. 3, 523.
3. Krogh, A., *Skand. Arch. Physiol.*, 1904, xv, 328.
4. Panizza, B., *Ann. sc. nat. zool.*, 1845, iii, series 3, 230.
5. Couvreur, E., *J. physiol. et path. gén.*, 1909, xi, 590.

EXPLANATION OF PLATE 29.

FIG. 1. Section of normal turtle lung showing thin walled respiratory membrane. Magnification × 240.

FIG. 2. Section of lung taken from turtle killed by exposure to warm oxygen. Magnification × 240. The photograph shows a characteristic hemorrhagic area with extravasated nucleated red blood corpuscles.



(Faulkner and Binger: Oxygen poisoning in cold blooded animals.)

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VII. On the Particulate Nature of Bacteriophage.

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(Received for publication, January 3, 1927.)

So called bacteriophage, as ordinarily obtained by filtration of broth cultures of susceptible bacteria after the completion of lysis, represents a heterogeneous mixture containing in addition to the active principle, the unaltered ingredients of the nutritive medium, products of bacterial metabolism, complex materials synthesized by bacteria, and liberated into the medium as the result of lysis of bacteria, etc.

Since at least a part of these substances is present in true solution, and since the active principle in all probability is carried by the particles of colloidal size (1), dialysis and differential ultrafiltration may offer means of eliminating from the filtrate the most highly dispersed and soluble ingredients. This is particularly advisable since we have found that such ingredients of the medium as inorganic salts, for example, may greatly increase the deleterious effect of various reagents which might be used in further purification of the active principle (2, 3). Numerous attempts to dialyze the active filtrates have given conflicting results (4–10). Not only have the findings differed when investigators employed membranes prepared of different material, but even when membranes of similar nature were used. Thus, for instance, Stassano and de Beaufort (11) found that phage passes freely through the membranes prepared from solutions of collodion in acetic acid, though such membranes were impervious to enzymes. Bechhold (6), as well as Villa (12), on the other hand, found that phage was kept back completely, even by such comparatively permeable membranes as those prepared from a 2 per cent solution of collodion in acetic acid. Our own findings show that even the more impervious membranes, up to 7 per cent and over, still allow diffusion of an appreciable fraction of the lytic agent.

However, when lytic filtrates were dialyzed through collodion membranes against fresh distilled water under osmotic pressure, it was found that while in general the rate of diffusion of phage was greater with the more permeable membranes, in no instance was it possible to observe a complete diffusion of the active principle. In all cases only a fraction of the active principle placed inside the membrane could be recovered in the dialysate, irrespective of the length of time during which dialysis was continued. So that if the dialysate was collected daily and replaced by fresh distilled water, after a few days the active principle no longer appeared in the outer fluid, while the inner fluid continued to show the presence of the bulk of the original active agent.

The following experiment is selected to illustrate this phenomenon.

Dialysis of Phage through Collodion under Osmotic Pressure.

Membranes of cylindrical shape were prepared from collodion dissolved in the mixture of alcohol and ether (13). By careful control of the initial amount of collodion used, the size of the surface over which it was spread, the time during which the solvents were allowed to evaporate from the film of collodion prior to its immersion in water, and the rate at which this evaporation of solvents was allowed to proceed, it was possible to prepare membranes of desired permeability. Membranes which were prepared under similar conditions were fairly comparable to one another in their permeability to water. The latter was determined in terms of time necessary for a given amount of water under constant pressure to pass through a unit of surface of the membrane suspended in air (13). By this procedure it was possible to check the relative permeability of membranes prepared and to select for duplicate experiments comparable membranes. Each membrane thus selected was filled with and suspended in water, and sterilized in the autoclave.

One of these membranes, allowing passage of 0.1 cc. of water under a pressure of 10 cm. of mercury over $\gtrless$ 44 sq. cm. of surface in 30 seconds, received 30 cc. of the Berkefeld filtrate of a lysed broth culture of *B. coli* (*coli* phage), was mounted on and suspended by means of a rubber stopper (through which was inserted a long glass tube 5 mm. in diameter) in a sterile receptacle containing 2000 cc. of sterile distilled water. After 24 hours of dialysis, the level of the water in the

Protocol I.

Dialysis of Phage under Osmotic Pressure.

		Duration of dialysis in days										Controls	
		0	1	2	3	4	5	6	8	10	12	15	Original fluid (inside)	Residue after dialysis (inside)
Osmotic pressure (about) in cm..		0	64.5	89	113.5	115.0	118.5	112.5	104.5	94	82	65		
Results of titration of lytic activity in cc.	10^{-10}	−	−	−	−								−	−
	10^{-9}	−	−	−	−								+	+
	10^{-8}	−	−	−	−								+	+
	10^{-7}	−	−	+	−								+	+
	10^{-6}		−	+	+									
	10^{-5}		+	+	+	−	−							
	10^{-4}		+	+	+	−	−							
	10^{-3}					+	+	−	−	−	−	−		
	10^{-2}					+	+	−	−	−	−	−		
	10^{-1}					+	+	+	−	−	−	−		
Number of units of phage dialyzed per day			2×10^{9}	2×10^{10}	2×10^{9}	2×10^{6}	2×10^{6}	2×10^{4}						
Total number of units of phage			$\gtrless 2 \times 10^{10}$										$\gtrless 3 \times 10^{10}$	$\gtrless 3 \times 10^{10}$* $\gtrless 1 \times 10^{10}$†

+ = lysis. − = no lysis.

*By titration. †By calculation.

glass tube rose to the height of 64½ cm. At this time the fluid in the outer receptacle was removed and replaced by fresh, sterile, distilled water. This first fraction of dialysate was titrated for its lytic activity by Appelmans' method, and the results of the titration were recorded, as shown in Protocol I. After the expiration of a second period of 24 hours, the osmotic pressure was recorded, the dialysate was again removed and titrated (Protocol I, 2), and the outer container was again filled with fresh, sterile, distilled water.

A similar procedure was followed at intervals indicated in Protocol I for 15 days, at the end of which time the experiment was terminated and a sample of the solution inside the collodion bag was titrated, in addition to the sample of the outer fluid.

It is apparent that over 50 per cent of the active principle was lost into the outer fluid during the first 5 days of dialysis, but none came through after this.[1]

These findings suggest at first glance that the particles which carry the active principle might not be uniform in size, and that the larger particles were kept back, while the smaller ones came through. However, since lytic filtrate contains a great deal of colloidal material (notably proteins), it is also possible that the changes in the rate of diffusion of phage were caused by a gradual diminution in the permeability of the membrane, due to adsorption of these substances (23). While regularity of the rate of change in osmotic pressure throughout the experiment would in itself suggest that there was no abrupt change in the permeability of the membrane about the 5th day of dialysis, still this possibility had to be considered before drawing conclusions as to lack of uniformity in the size of particles carrying active principle, particularly as earlier publications lead one to believe that the active principle consists of particles of approximately uniform size, approaching 20 $\mu\mu$ in diameter (11, 6, 14, 8).

Possibility of the Partial Occlusion of the Pores of the Membrane.

In order to exclude this possibility, the following experiment was performed.

30 cc. of a fresh lytic filtrate was dialyzed through one of two collodion membranes, the permeability of which was similar to that of the membrane used in the

[1] The titration of the activity of the residue does not indicate this loss because of the tenfold method of dilution in titration.

preceding experiment. The outer fluid was changed daily and was found to be free from active principle from the 7th day on. On the 12th day[2] 20 cc. of the residue within the dialyzing bag was removed and placed into a second bag. At the same time, 20 cc. of fresh lytic filtrate was introduced into the first bag. Both were suspended in distilled water and dialysis was continued for 4 days, as illustrated in Protocol II.

These results indicate that the residue remaining inside the dialyzing membrane does not diffuse through a fresh membrane of similar

Protocol II.

Permeability of the Membrane after the 6th Day of Dialysis.

Dialyzing Bag I 30 cc of fresh lytic filtrate		
Titer of the dialysate in cc.	1st day	1×10^{-4}
	2nd "	1×10^{-6}
	3rd "	1×10^{-7}
	5th "	1×10^{-4}
	6th "	1×10^{-2}
	7th "	Negative
	8th "	"
	9th "	"
	10th "	"

On the 12th day 20 cc. of the fluid inside the bag was removed and replaced by 10 cc. of fresh lytic filtrate. The fluid removed was placed into Bag II.

Dialyzing Bag I			Dialyzing Bag II
Residue from dialysis + 10 cc. of fresh lytic filtrate			20 cc. of the residue from Bag I
Titer of the dialysate in cc.	1st day	1×10^{-4}	Negative
	2nd "	1×10^{-4}	"
	3rd "	1×10^{-5}	"
	4th "	1×10^{-3}	"

permeability, and that, on the other hand, the first membrane which held back a portion of the active principle after 5 days of dialysis is permeable to the fresh lytic filtrate. The inference is that the failure of the active principle to appear in the dialysate after 5 days (Protocol I) is not due to the change in permeability of the membrane, but

[2] 2 days were allowed to elapse in order to obtain the final check on the titration of the dialysate removed on the 10th day.

to the fact that particles carrying the active agent are not uniform in size, and that only smaller particles could pass through the pores of the membrane used in this experiment.

If the above inference is correct—that is, if the fraction of the active principle which dialyzed through is carried by particles of smaller size—then the active principle present in dialysate must pass freely through another membrane of similar permeability. In view of the fact that the dialysate, as obtained in the preceding experiment, is very highly diluted and does not lend itself therefore to such an experiment as the one just outlined, we undertook to fractionate the lytic filtrate by ultrafiltration instead of dialysis as before.

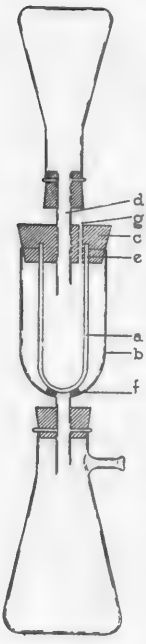

FIG. 1.

The preparation of the membranes and the procedure followed in ultrafiltration are as follows:

Alundum thimbles (Norton RA 360 135 mm. × 45 mm.), glazed to our specifications for a distance of 2 cm. from the rim on the inside surface and for a distance of 2.5 cm. on the outer surface, were used to support a collodion membrane. The latter consists of soluble cotton of any desired concentration, in glacial acetic acid, and is deposited on the inner surface of the alundum under a vacuum. For this purpose a clean (preferably freshly incinerated) thimble (*a*) is filled with water and adjusted to a Gooch funnel of proper size, (*b*) by means of a rubber stopper (*c*) having a small hole in the center (*d*) and a circular groove fitting the ridge of the thimble (*e*). The funnel is then placed over a filtration flask connected with the vacuum pump. On the bottom of the funnel rests a thin, perforated, rubber disk (*f*), protecting the lower end of the thimble against possible injury due to pressure against the funnel (Fig. 1). After all free water is removed by suction, and only traces of water remain adsorbed on the wall of the thimble, a solution of collodion is admitted to the thimble through the hole in the center of the stopper until the thimble is filled with collodion. The suction is maintained for 60 seconds so as to remove all the air from the pores of the thimble and to secure an even membrane without flaws. As soon as connection with the vacuum pump is discontinued, the collodion is quickly poured out of the thimble while the latter is being rotated, to secure an equal thickness of the membrane. During this manipulation care must be taken in handling the thimble not to touch the unglazed surface, to prevent the

production of flaws in the membrane due to uneven evaporation of acetic acid. When no more collodion runs off (2–3 minutes) the thimble is placed under a water faucet and quickly filled with water in order to solidify the collodion. Special precautions must be taken not to wet the outer surface of the thimble before filling it with water, as deposition of water droplets on the outer surface drives the air contained in the clay inward and results in the production of air pockets between the clay wall and the membrane. When the entire thimble is filled with water it may be dropped into a vessel containing running water, for the purpose of washing out the acetic acid. The latter process can be hastened by placing the thimble into the funnel and washing it under negative pressure with warm water.

Protocol III.

Permeability of Collodion to Whole Phage and to Ultrafiltered Fraction Respectively under Negative Pressure.

Titer in cc.	Original phage (control)	Ultrafiltrate A	First washing B	Second washing C	Third washing D	Residue E	Fourth washing F	Ultrafiltrate from A G	Ultrafiltrate from B H
10^{-10}	−					−			
10^{-9}	+					+			
10^{-8}	+					+			
10^{-7}	+		−			+			−
10^{-6}	+	−	+			+			+
10^{-5}	+	+	+			+		−	+
10^{-4}	+	+	+	−		+		+	+
10^{-3}	+	+	+	+		+		+	+
10^{-2}	+	+	+	+	−	+	−	+	+
10^{-1}	+	+	+	+	+	+	+	+	+
10^{-0}	+	+	+	+	+	+	+	+	+

+ = lysis. − = no lysis.

If the removal of acid is not complete, a portion of the active agent is adsorbed on the membrane during ultrafiltration. Similar loss of the active agent is observed if, instead of adequate removal of acid, the latter is merely neutralized with ammonia (6). It is preferable, therefore, to remove the last traces of acid by adequate washing and electrodialysis (21). Membranes prepared in this way may be used repeatedly, provided they are kept under water when not in use and are sterilized by immersion in alcohol.[3]

[3] In another connection Dr. Hetler and myself have used this ultrafilter for continuous filtration of large amounts of material by inserting a tube connected with an inverted container through the hole (*d*) in the center of the stopper. In this case a second hole (*g*) which serves to admit the air operates as an automatic regulator for the continuous filling of the thimble from the reservoir above.

Passage of Phage through Collodion under Pressure.

20 cc. of fresh bacteriophage solution was forced, under vacuum of 38 cm., through a membrane of 7 per cent soluble cotton in acetic acid. The ultrafiltrate thus obtained was collected and titrated to determine the concentration of the lytic agent in it (Protocol III, A).[4] The residue which failed to pass through the membrane was diluted with 20 cc. of distilled water and subjected to a second filtration through the same membrane. The ultrafiltrate was again collected and titrated (Protocol III, B), and the residue again diluted with 20 cc. of water and forced through the membrane for the third time. As will be seen from the protocol (Protocol III, C), the ultrafiltrates obtained at this time, as well as upon a further repetition of the procedure (Protocol III, D), contained only faint traces of active agent. The residue in the thimble was again diluted to 20 cc. with water, and a sample taken for titration showed that the residue still contained the bulk of the lytic agent (Protocol III, E). The contents of the thimble were then subjected to filtration through the same membrane for the fifth time, and the ultrafiltrate titrated (Protocol III, F). At this time the ultrafiltrate collected in the first filtration (Protocol III, A) was added to the residue in the thimble, and subjected to ultrafiltration. It will be seen (Protocol III, G) that the fluid which came through the membrane at this time contained a comparatively high concentration of active principle.

At the same time the ultrafiltrate obtained from the first washing of the residue (Protocol III, B) was forced through a fresh 7 per cent membrane and the ultrafiltrate thus obtained was titrated. In this case (Protocol III, H), the ultrafiltrate showed no diminution in activity, as compared with Solution B.

The results of this experiment confirm those obtained earlier, in that they show that only a portion of lytic agent passes through the membrane, that the residue which is held back contains the bulk of the active principle, and yet it can be washed repeatedly without loss of any more active substance into the filtrate. On the other hand, the filterable portion of the active principle passes freely when added to this residue, or when filtered through a fresh membrane.

These findings suggest that the active principle is carried by the particles, which may differ in size, and therefore that only a part of the active principle which is adsorbed on the smallest particles may pass through a membrane of given porosity.

[4] Titrations were made both by the method of serial dilution and by the plaque method. Incidentally it was observed that ultrafiltrates consistently yielded plaques considerably smaller in size than those produced by the agent remaining on the filter.

These experiments were repeated a number of times with the lytic agents active against *B. coli*, *B. dysenteriæ* Shiga, *B. pestis caviæ*, and staphylococcus, and in all cases identical results were obtained. It was found that with all lytic filtrates the proportion of filterable fraction was approximately the same, irrespective of the origin of bacteriophage or bacterial substratum. This observation suggested that nutrient broth itself served as a principal source of the particles capable of passing through the ultrafilter. The validity of this inference seems to be supported by the following experiment, in which the addition of sterile broth to the non-filterable residue of the phage resulted in renewed passage of the active principle through the membrane.

Effect of Broth on the Filterability of Phage.

A portion of lytic filtrate was subjected to ultrafiltration and six repeated washings with water as previously (Protocol III). The ultrafiltrates thus obtained were collected and subsequently titrated for lytic activity (Protocol IV, A).

At the same time another portion of the same phage was subjected to ultrafiltration and three repeated washings with water. At this time the residue in the thimble was diluted to 20 cc. with sterile broth (pH = 7.4) and subjected to ultrafiltration. Following this it was washed with water as before two more times (Protocol IV, B). As a control a third portion of the phage was filtered through a third membrane in exactly the same manner as the second but instead of broth in the fourth washing a buffer mixture of pH = 7.4 was used (Protocol IV, C).

It appears that when sterile broth was added to the residue on the filter (Protocol IV, B), a portion of the phage which was adsorbed on the coarser particles became detached and came through the collodion with the filterable portion of the broth, thus apparently assuming a more dispersed state. This conclusion, however, is valid only if it can be shown that the broth did not modify the membrane itself. This was actually done. When a clean ultrafilter was saturated with broth prior to the filtration of the residue, the latter did pass through to some extent. However, if such an ultrafilter was washed free of broth, it became again impermeable to the phage residue, which would not have been the case if broth had been adsorbed on the membrane in a manner analogous to that of gelatin in the experiments of Hitchcock (23).

Protocol IV.

Effect of Broth on the Rate of Ultrafiltration of Phage.

	Titer of activity in cc.										
	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	10^{-0}
Original phage	−	+	+	+	+	+	+	+	+	+	+
7 per cent membrane A — Ultrafiltrate				−	−	+	+	+	+	+	+
7 per cent membrane A — First washing with water				−	+	+	+	+	+	+	+
7 per cent membrane A — Second washing with water					−	+	+	+	+	+	+
7 per cent membrane A — Third washing with water						−	−	+	+	+	+
7 per cent membrane A — Fourth washing with water							−	−	+	+	+
7 per cent membrane A — Fifth washing with water								−	−	+	+
7 per cent membrane A — Sixth washing with water								−	−	−	+
7 per cent membrane B — Ultrafiltrate				−	−	+	+	+	+	+	+
7 per cent membrane B — First washing with water				−	−	−	+	+	+	+	+
7 per cent membrane B — Second washing with water					−	−	−	+	+	+	+
7 per cent membrane B — Third washing with water						−	−	−	+	+	+
7 per cent membrane B — Fourth washing with *broth*						−	−	+	+	+	+
7 per cent membrane B — Fifth washing with water						−	−	−	+	+	+
7 per cent membrane B — Sixth washing with water						−	−	−	+	+	+
7 per cent membrane C — Ultrafiltrate					−	−	+	+	+	+	+
7 per cent membrane C — First washing with water						−	−	+	+	+	+
7 per cent membrane C — Second washing with water							−	−	+	+	+
7 per cent membrane C — Third washing with water							−	−	−	+	+
7 per cent membrane C — Fourth washing with *buffer* ($pH = 7.4$)							−	−	+	+	+
7 per cent membrane C — Fifth washing with water									−	−	+
7 per cent membrane C — Sixth washing with water									−	−	+

DISCUSSION.

Direct (20), as well as indirect (6) microscopic examination of lysed cultures of bacteria, and especially the behavior of lytic filtrates in high dilutions (1) and their ability to cause the appearance of discrete foci of lysis in bacterial cultures on solid media have led to the generally accepted view that the active principle of transmissable lysis (bacteriophage) is present in filtrates of lysed cultures in the form of discrete particles.

By means of ultrafiltration through membranes of known porosity several authors (6, 11, 14) have independently estimated the size of these particles as being approximately 20 $\mu\mu$ in diameter, and consequently having a calculated mass of about $\frac{4}{3}\pi\ 10^{-18}$ gm. (15). Moreover, similar figures were obtained when the size of particles of bacteriophage was estimated by optical methods. Thus, von Angerer (16) found, on the basis of his study of the turbidity of filtrates, that the diameter of particles approximates 20–35 $\mu\mu$. This uniformity of the size of the particles, as found by different investigators and by means of different methods, has in itself appeared to many to be a strong indication in favor of the conception that the particles represent the units of an autonomous organized virus, as originally suggested by d'Hérelle.

However, some experiments, particularly those showing that the active principle is capable of spreading from the focus radially (16), independently of the multiplication of bacteria, and independently of gravity, and that the rate of its spread is conditioned by the density of the medium (1) seem to militate against ready acceptance of such a view.

If the autonomous particulate nature of the active principle is accepted, such spreading must be accounted for either by postulating for the particles the power of locomotion (17), or by assuming that they secrete a diffusible enzyme (18). But independent locomotion, even if claimed to exist (17), (whether it be ameboid or due to the activity of a propelling mechanism, such as flagellum or cilia) would explain only the transport of a given particle in one plane at the time, whereas the active principle spreads from the focus in all directions

simultaneously (16). The secretion of the active enzyme has actually been suggested by d'Hérelle (18), but the evidence presented by him was later found to be inadequate (19).

On the other hand, such radial spreading could be easily accounted for by a working assumption (3) that the observed particulate distribution of phage is only apparent and is due to its ready adsorption on coarser colloidal particles of the medium. That such an assumption is more valid has been suggested by our experiments in which the number of particles endowed with specific activity of phage in a given volume of a filtrate was altered, depending on changes in the degree of dispersion of colloids in the medium (1). Experimental data presented in this paper strengthen this conception further by showing that the particles present in filtrates of lysed cultures of bacteria and endowed with properties of the phage are not uniform in size. Only that portion of the active agent which is carried by the smallest particles was found capable of passing through semipermeable membranes, while the bulk of it failed to pass through even on repeated washing under pressure. While this failure to pass through a membrane is not of itself sufficient evidence to indicate the size of the particles, under the conditions of our experiments, where the effect on the membrane was excluded by proper controls (Protocol III, G and H), failure of the active residue to pass the filter appears to indicate that it is composed of particles too great in size to pass through the largest pores of the ultrafilter employed.

In this connection, it is of interest to note that in our earlier experiments (19), it was found that only a portion of the active agent was carried down with the precipitate produced by the addition of an excess of alcohol at 7°C., and the supernatant fluid usually retained enough active agent to make 1×10^{-5} cc. of it capable of causing lysis of susceptible bacteria. If the rate of precipitation was increased by the addition of electrolytes or by an increase in temperature, the precipitate carried down more of the active principle. It would appear that in this case also only larger particles were precipitated by the alcohol at 7°C., and the supernatant fluid represented that portion of the phage which corresponds almost quantitatively to that fraction which passed the ultrafilter in the experiments reported in this paper.

The fact that active principle can thus be shown to be distributed in the medium in the form of particles of different size does not necessarily deny its autonomous particulate nature, since the phage can conceivably be assumed to be a pleomorphic virus. However, the fact that the addition of broth to the non-filterable residue (presumably composed of units of the agent of larger size) allows it to pass through the membrane which held it back before the addition of broth, is contrary to such an assumption, provided, as we have shown, the permeability of the membrane was not altered by the addition of broth.

Incidentally, in the light of these findings it seems likely that used Berkefeld candles which might have retained some active principle might not give it up into the filtrate if water is passed through them, and yet, if broth is passed instead of water, the filtrates may show some activity due to the detachment of the phage from the coarser particles by the broth. Such an effect of broth would explain the occasional findings of several workers (22) who believe they have caused spontaneous production of phage by filtration of bacterial cultures, heated lytic filtrates, and even sterile broth—substrata assumed by them to be free from bacteriophage before filtration.

SUMMARY AND CONCLUSIONS.

When filtrates of lysed cultures (bacteriophage) are subjected to prolonged dialysis under osmotic pressure against water, the presence of the lytic agent can be detected outside the membrane only during the first few days. The residue remaining inside the membrane contains the bulk of the original lytic agent, and yet it is no longer capable of diffusing into the outer solution.

The interruption of diffusion is shown not to be due to any alteration in the permeability of the membrane. Moreover, the residue fails to diffuse through a fresh membrane of similar permeability, while the dialyzed portion of the phage passes quantitatively through a new membrane. When ultrafiltration under pressure was substituted for dialysis, the residue on the filter could be washed repeatedly with water without giving off into the filtrate any more active agent. However, if broth was substituted for water, a renewed diffusion of the active agent resulted.

These results are interpreted as indicating that the colloidal particles present in the lytic filtrates (and apparently endowed with properties of bacteriophage) do not represent autonomous units of the active agent, but merely serve as a vehicle on which the agent is adsorbed. They vary in size within limits wide enough to permit fractionation by means of ultrafiltration. When the coarser particles retained by the ultrafilter are washed with broth, some of the active agent is detached from its coarse vehicle particles. This agent, now more highly dispersed, is capable of passing the filter which held it back previously.

Preparation of a simple ultrafilter used in these experiments is given in detail.

BIBLIOGRAPHY.

1. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483; *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 315.
2. Bronfenbrenner, J., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 187.
3. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1926, xliii, 71.
4. d'Hérelle, F., *Compt. rend. Soc. biol.*, 1925, xciii, 509.
5. Zoeller, Chr., and Manoussakis, *Compt. rend. Soc. biol.*, 1925, xciii, 1091.
6. Bechhold, H., and Villa, L., *Z. Hyg. u. Infectionskrankh.*, 1926, cv, 601.
7. Bürgers and Bachman, W., *Z. Hyg. u. Infectionskrankh.*, 1924, ci, 350.
8. Hajós, K., *Z. Immunitätsforsch., Orig.*, 1923, xxxvii, 147.
9. Asheshov, I. N., *Compt. rend. Soc. biol.*, 1925, xciii, 643, 644.
10. Doerr, R., and Zdansky, E., *Z. Hyg. u. Infectionskrankh.*, 1923, c, 79.
11. Stassano, H., and de Beaufort, A.-C., *Compt. rend. Soc. biol.*, 1925, xciii, 1378.
12. Villa, L., *Folia clin.*, 1926, i, 52.
13. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926, ix, 351.
14. Prausnitz, C., *Klin. Woch.*, 1922, i, 1639.
15. Prausnitz, C., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 148.
16. von Angerer, K., *Arch. Hyg.*, 1924, xcii, 312.
17. Kabelik, J., and Kukula, K., *Compt. rend. Soc. biol.*, 1925, xcii, 1058.
18. d'Hérelle, F., Le bacteriophage. Son rôle dans l'immunité, Monographies de l'Institut Pasteur, Paris, 1921, 95.
19. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 419.
20. d'Hérelle, F., The bacteriophage. Its rôle in immunity, translation by Smith, G. H., Baltimore, 1922, 113.
21. Bronfenbrenner, J., *J. Gen. Physiol.*, 1926, x, 23.
22. Otto, R., and Winkler, W. F., *Deutsch. med. Woch.*, 1922, xlviii, 383; Weinberg, M., and Aznar, P., *Compt. rend. Soc. biol.*, 1922, lxxxvi, 833; lxxxvii, 136; Hajós, K., *Z. Immunitätsforsch., Orig.*, 1923, xxxvii, 147; Yasaki, Y., *Sei-I-Kwai Med. J.*, 1925, xliv, No. 3, 1.
23. Hitchcock, D. I., *J. Gen. Physiol.*, 1926, ix, 755.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VIII. The Mechanism of Lysis of Dead Bacteria in the Presence of Bacteriophage.

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(Received for publication, January 15, 1927.)

With but few exceptions (1, 2), those engaged in the study of bacteriophage agree that one of the essential features of transmissible lysis of bacteria is the fact that the lytic principle undergoes an increase in activity exclusively in the presence of living and actively multiplying bacteria. Without active growth of susceptible bacteria, there is no reproduction of lytic agent and no observed bacterial lysis. Although dead susceptible bacteria readily adsorb the lytic agent, they do not dissolve (3–7). However, Gratia and Rhodes (8) observed that dead staphylococcus may be slowly lysed by the bacteriophage, and that the concentration of the latter in solution probably increases during this process. If live staphylococci are present simultaneously, the lysis of the dead bacteria is more rapid. The lytic effect of live on dead staphylococcus was observed by them also in the absence of bacteriophage[1] and was assumed by them to be due to the utilization of dead bacteria by the live in the process of nutrition (10). The relation of this phenomenon to the one described earlier by these authors (8) was not made clear. A year later Twort (11) independently observed the lysis of dead staphylococcus in the presence of bacteriophage and live bacteria, and suggested that this was made possible through the activation of the bacteriophage by some auxiliary substance contributed by the live bacteria. Because of the importance of these observations on the question of the mechanism of transmissible lysis, we undertook to study it.

[1] A similar observation has also been made recently by Duran-Reynals (9).

The Lysis of Dead Bacteria.

An 18 hour agar slant culture of *Staphylococcus aureus* "G" was suspended in saline, sealed in an ampoule, and submerged for 1 hour in a water bath at 60° C. to kill the bacteria. 0.3 cc. of this suspension of dead bacteria was placed into each of a series of tubes containing 9 cc. of broth each, and an homologous bacteriophage alone,[2] or together with live staphylococci, was added to some of these tubes, as indicated in Protocol I.

The degree of turbidity resulting, as well as a subsequent increase or decrease in turbidity, was measured by comparison with a specially prepared set of standards in which the turbidity gradually increased from the first to the tenth tube. The figures expressing the results of this and later experiments indicate the serial numbers of the tubes in this arbitrary set of standards to which the turbidity in a given tube was comparable.

Protocol I.

Lysis of Dead Staphylococci.

Tube No.		1	2	3	4	5	6
Broth in cc.		9	9	9	9	9	9
Suspension of dead staphylococci in cc.		0.3	0.3	0.3	0.3	0	0
" " live " " "		0	0	0.1	0.1	0.1	0.1
Phage in cc.		0	0.1	0	0.1	0	0.1
Salt solution		0.7	0.6	0.6	0.5	0.9	0.8
Incubated at 37°C.							
Turbidity of resulting suspensions	Immediately	4	4	5	5	1	1
	After 4 hrs.	4	4	8	4	8	0
	" 24 "	4	4	10+	1	10+	0

It will be seen from Protocol I that the suspension of dead staphylococci did not undergo lysis by the bacteriophage alone (Tube 2), but when both live staphylococci and the phage were present simultaneously (Tube 4), dead staphylococci underwent almost complete lysis, as shown by the decrease in turbidity of the suspension from that corresponding to Tube 5 of the standard scale to that corresponding to Tube 1—the lowest tube in the scale.

In attempting to repeat this experiment, using other bacteria than staphylococcus (*B. coli* and *B. dysenteriæ*), we failed to obtain clear-

[2] Unless otherwise stated, the bacteriophage used in these experiments was taken out of the stock kept on ice several weeks.

cut results. This explains the negative results obtained by Doerr and Grüninger (7), who attempted to produce dissolution of dead colon bacillus in the presence of corresponding bacteriophage and of live bacteria. Moreover, we found that even with staphylococcus, reproducible results can be obtained only if the relative bulk of dead bacteria used is taken into account, as will be shown in the following experiment.

Effect of Variations in Relative Concentration of Phage and of Dead Bacteria.

Two series of eight tubes each received equal amounts of broth (5 cc.). Each of the tubes of the first series (A) received 0.1 cc. of antistaphylococcus phage diluted in broth 1:1000; and the tubes of the second series (B) each received 0.1 cc. of the same phage diluted 1:100,000. Following this, the first four tubes of each series received 0.1 cc. each of bacterial suspension containing 80 million of live staphylococci per cc., and gradually decreasing amounts of a suspension of dead staphylococci containing 220 million bacteria per cc.—the first tube of each series receiving 5 cc. of this suspension, the second 0.5 cc., the third 0.05 cc. respectively. The fourth tube served as control and received no dead bacteria. The fifth, sixth, and seventh tubes of each of the series received respectively 5 cc., 0.5 cc., and 0.05 cc., of the suspension of dead bacteria only and no live bacteria, and the last tube (No. 8) of each series received no bacteria at all. The volume of liquid in all the tubes was brought to 10.2 cc. with physiological salt solution, and the whole was kept for 24 hours at 37°C. Changes in turbidity due to lysis of bacteria were recorded, as indicated in Protocol II, and after 24 hours' incubation all the tubes were placed for 30 minutes into a water bath kept at 56°C., and the phage titer of each mixture was determined by the method of Appelmans.

This experiment indicates that both in the series where the initial titer of the phage was 1×10^{0}cc. (Protocol II, B, Tube 8), and where it was 1×10^{-1} cc. (Protocol II, A, Tube 8), dead bacteria alone adsorbed the entire phage (Tubes 5, 6, and 7 of each series), the rate of adsorption apparently depending on the number of dead bacteria present. When the concentration of dead bacteria was comparatively low, the adsorption of phage was so slow that when live bacteria which had been added began to multiply, there was sufficient phage left free in solution to produce its usual effect on the live bacteria and to regenerate. In the case where the initial concentration of phage was lower (Series B), the regeneration of phage took place only in Tube 3, containing the lowest number of dead bacteria. In Series A,

Protocol II.

Effect of the Number of Dead Bacteria Present in the Mixture on the Regeneration of Phage.

	A								B							
Tube No.	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Broth in cc.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Phage 1:1000 in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1								
" 1:100,000 in cc.									0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Suspension of live staphylococci in cc.	0.1	0.1	0.1	0.1					0.1	0.1	0.1	0.1				
" " dead " " " "	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0
Salt solution in cc.	0	4.5	5	5	0.1	4.6	5.1	5.1	0	4.5	5	5	0.1	4.6	5.1	5.1
Incubated at 37°C. for 24 hrs. The changes in turbidity during the incubation were recorded at intervals, as indicated below.																
Turbidity: Immediately	10	7	5	1	10	7	5	0	10	7	5	1	10	7	5	0
Turbidity: After 4 hrs.	10+	7	4	1−	10	7	5	0	10+	10	5	1−	10	7	5	0
Turbidity: " 24 "	10+	5	2	0	10	7	5	0	10+	10+	3	0	10	7	5	0
All tubes were heated at 56°C. for 30 min. and the titer of phage in each determined.																
Phage titer in cc.*	0	10^{-6}	10^{-7}	10^{-7}	0	0	0	10^{-1}	0	0	10^{-7}	10^{-7}	0	0	0	10^{0}

* The sign 0 = no phage present in 1 cc. of the mixture.

Variation between 10^{-6} cc. and 10^{-7} cc. in the final titer is due to particulate distribution of phage (25).

where the initial concentration of phage was ten times greater, the regeneration of the phage took place both in Tube 3 and in Tube 2. It is evident that the absence of regeneration of phage in Tube 1 of Series A, and in Tubes 1 and 2 of Series B was due to the fact that the phage present in the mixtures was so completely taken up by the dead bacteria that by the time live bacteria had begun to multiply actively (which is essential for the regeneration of phage) after a period of initial lag, there was no free phage left in the mixture. As a result of this, the bacteria in these tubes remained intact, as is evidenced by the fact that the original turbidity of the contents increased slightly, due to multiplication of the live bacteria. On the contrary, in such tubes of each series as exhibited regeneration of phage in the presence of dead bacteria (Tubes 2 and 3 of Series A, and Tube 3 of Series B), the initial turbidity decreased, due to dissolution of dead bacteria.

Specificity of Lysis of Dead Bacteria.

It was seen in the preceding experiments that while phage alone does not cause lysis of dead bacteria, the latter are lysed if phage and live bacteria are present simultaneously and if the concentration of dead bacteria is kept sufficiently low not to interfere with the process of regeneration of the phage. Another condition essential for the lysis of dead bacteria is that the dead and live bacteria be of the same species. This requirement has already been indicated by Twort (11), and we have been able to confirm it. Dead colon bacilli or dead dysentery bacilli were not lysed in the presence of live staphylococcus and staphylococcus phage.

Time Relation of the Lysis of Live and of Dead Bacteria.

The preceding experiments show that live bacteria contribute some specific active principle necessary for the production of lysis of dead bacteria. Twort (11) has suggested that bacteria supply some sort of a complementary substance which activates the phage and disappears as the phage ages. In order to see whether such a substance is present, and to determine more accurately at what stage of lysis of live bacteria it first appears and how long its activity continues, the following experiment was performed.

A series of six tubes containing 10 cc. of broth each received 0.1 cc. of a suspension of live staphylococcus and 0.1 cc. of phage, and were placed in the incubator at 37°. The first tube of the series received at the same time 0.2 cc. of a suspension of heat-killed staphylococcus. At intervals of 2, 4, 6, etc., hours after

Protocol III.

Dissolution of Dead Bacteria Added during and after the Completion of the Lysis of Live Bacteria in the Presence of Bacteriophage.

	Test proper						Controls				
Tube No.	1	II	3	4	5	6	7	II	9	10	11
Broth in cc.	10	10	10	10	10	10	10	10	10	10	10
Suspension of live staphylococci in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0
Phage (filtrate) in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0	0.1	0	0.1	0
Incubated at 37°C.											
Interval before addition of the suspension of dead bacteria in hrs.	0	2	4	6	24	48					
Suspension of dead bacteria in cc.	0.2	0.2	0.2	0.2	0.2	0.2	0	0	0.2	0.2	0.2
Turbidity: Immediately	4						1	1	4	3	3
2 hrs. later	4	4					1	1+	4	3	3
4 " "	5	3	4				5	1	6	3	3
6 " "	3	2	2	3			10	0	10	3	3
24 " "	1	2	6	1	3		10+	0	10+	3	3
30 " "	1+	3	6	1+	2−		10+	1	10+	3	3
48 " "	6	6	7	7	3*	7*	10+	4	10+	3	3
72 " "					6*	6*	10+	6	10+	3	3

* Final results in these tubes are somewhat obscured, due to increase in turbidity caused by the multiplication of the resistant bacteria, but the lysis of dead bacteria has taken place even in these tubes, as suggested by the temporary diminution in turbidity in Tubes 5 and 6.

the beginning of the experiment, other tubes of the series each received in turn 0.2 cc. of the same suspension of dead bacteria. The progress of lysis of live, as well as of dead bacteria, was followed and is recorded in Protocol III in terms of turbidity, by comparison with a standard scale, in which the higher numbers indicate greater turbidity. A fall in turbidity below that of the control tube (No. 11), containing dead bacteria alone, indicates the lysis of dead bacteria. It will be

seen that the initial fall in turbidity in all the tubes of the series (Nos. 1 to 6) is followed by a subsequent increase. This is due to the growth of resistants following the initial lysis of the susceptible live bacteria, so that the results in Tubes 5 and 6 are difficult to interpret on the basis of turbidity.

The results show that dead bacteria are dissolved when added to the live bacteria undergoing lysis, any time within 24 hours after the beginning of the experiment, and probably even later, irrespective of the stage at which the lysis of live bacteria may happen to be. Since the lysis of live bacteria under the conditions of the experiment was completed in the first 6 hours (see Tube 8), and since dead bacteria were dissolved when added even much later, it is evident that the actual lysis of live, is not essential for the dissolution of dead bacteria, and that the products of such lysis alone are capable of causing dissolution of dead bacteria.

However, we have observed that filtrates of lysed cultures which should thus contain these products are inactive against dead bacteria (Protocol I). It was suspected, therefore, that during filtration they might have been kept back by the filter, while phage was able to pass freely. If such were the case, then it would appear that the agent responsible for the dissolution of dead bacteria is distinct from the phage proper and can be separated from the latter because of the difference in their respective diffusibility.

In order to determine if such were the case, an attempt was made to interpose a semipermeable membrane between the live and dead bacteria during the lysis of the former to see whether this procedure would prevent the dissolution of the dead bacteria (12).

Separation of Lysed Cultures into Two Fractions by Means of a Semipermeable Membrane.

A series of cylindrical collodion membranes 15 mm. in diameter and 50 mm. long were prepared under carefully controlled conditions. After hardening in water, these membranes were tested for their relative permeability by measuring the time required for 0.1 cc. of water, under pressure of 10 cm. of mercury, to be forced through the membrane suspended in air. It was found that membranes allowing this amount of water to pass through in from 20 to 30 seconds were suitable for the experiment. A number of such selected collodion bags were filled with and suspended in water, and sterilized in the autoclave for 10 minutes, at 20 pounds pressure. While the autoclaving renders the membrane somewhat less permeable

Protocol IV.

Separation of Phage Proper from the Agent Responsible for the Lysis of Dead Bacteria by Dialysis.

			A			B			C			D		
			Inside		Outside	Inside		Outside	Inside		Outside	Inside		Outside
Broth in cc.			5		150	5		150	5		150	5		150
Phage " "			0.5			0.1					0.1			0.1
Live bacteria								0.1			0.1			
Dead "									0.1			0.1		
												0.1		
Duration of incubation in hrs.			0	5	24	0	5	24	0	5	24	0	5	24
Results	Gross lysis	Inside							0	0	0	0	Partial lysis	
		Outside				0	0	Lysis	0	0	Lysis			
	Phage titer in cc.	Inside	1×10^{-9}	1×10^{-8}	1×10^{-7}				0		1×10^{-8}			
		Outside	0	1×10^{-6}	1×10^{-7}				1×10^{-8}		1×10^{-9}			
	Bacteria in millions per cc.	Inside							163	161	165	267.5	29	96*
		Outside												

* These figures represent averages of several counts made on Breed plate or in a counting chamber.

to water, we have found by experience that bags selected and tested as stated above are uniformly permeable to bacteriophage. At the time of the experiment the dialyzing thimbles were removed from the water in which they had been sterilized, filled with measured amounts of sterile broth, and placed into suitable containers, with aseptic precautions. In each experiment four dialyzing thimbles were set up as follows:

I. In the first dialyzing unit a measured amount of antistaphylococcus bacteriophage was added to the broth inside of the dialyzing thimble, and a small portion of the resulting dilution of phage was immediately taken out for titration by the method of Appelmans. At the same time a small portion of the broth outside of the bag was likewise removed for titration, and the whole unit was placed in the incubator at 37°C. After an interval of 5 and 24 hours respectively, the titration of the fluid inside and outside the thimble was carried out, and it was found that the lytic agent dialyzed freely under these conditions ((Protocol IV, A).

II. In the second dialyzing unit 5 cc. of broth were placed inside and 150 cc. of broth outside the thimble as before, following which 0.1 cc. of antistaphylococcus bacteriophage was added to the broth inside the thimble, and 0.1 cc. of an 18 hour old culture of susceptible staphylococcus to the broth outside. The whole unit was placed in the incubator and observed at intervals. It was found that live bacteria placed outside the thimble underwent lysis (Protocol IV, B), just as they do when placed in direct contact with phage without the interposition of the membrane.

III. The third dialyzing unit was set up exactly as the preceding one, except that both live bacteria and the phage were placed outside, and on the inside only 0.1 cc. of a suspension of dead (heat-killed) staphylococcus. As the experiment proceeded in the incubator at 37°C., lysis of live bacteria took place outside the thimble, and the phage dialyzed into the thimble, where it was demonstrated by titration. However, the actual count of dead bacteria placed into the thimble remained unaffected throughout the experiment (Protocol IV, C).

IV. In the fourth dialyzing unit the phage was placed outside the dialyzing thimble and allowed to dialyze for 6 hours into the sterile broth inside. At this time 0.1 cc. of a suspension of live staphylococcus was added to the fluid inside the thimble, and a sample was removed for immediate count, which was found to be 1,000,000 bacteria per cc. Immediately following the removal of the sample, 0.1 cc. of a suspension of dead staphylococcus was added, and again a sample was removed. The bacterial count in this sample indicated the presence of 267,500,000 bacteria per cc. Thus, the initial mixture inside the thimble was composed of 1,000,000 live, and 266,500,000 dead bacteria per cc. From then on the bacterial count was repeated at intervals, and it was found that at the end of 5 hours it had fallen to 29,000,000 per cc., and at the end of 24 hours it had risen to 96,000,000 per cc., due to overgrowth of resistant bacteria. As the initial mixture contained 266,500,000 dead bacteria per cc., it is evident that at the end of 5 hours, practically 90 per cent of the dead bacteria had been dissolved.

These tests indicate that the phage responsible for transmissible lysis of live bacteria is easily diffusible, but that the agent liberated during the lysis of live bacteria and causing dissolution of dead bacteria does not pass through the membrane, so that dead bacteria remain unaffected if the active lysis of live bacteria takes place on the other side of the membrane, even though the bacteriophage can be demonstrated in abundance in the dialysate in which dead bacteria are suspended.

Protocol V.

Effect of Adsorption with Live Bacteria on the Phage Titer and Power to Dissolve Dead Bacteria of Lysed Staphylococcus Cultures.

	A						II
	Dissolution of dead staphylococcus						Titer of phage
Original lysed culture in cc.	0	5					1×10^{-6}
Supernatant fluid after the first adsorption in cc.			0	5			1×10^{-3}
Supernatant fluid after the second adsorption in cc.					0	5	1×10^{-1}
Broth in cc.	5	0	5	0	5	0	
Suspension of dead bacteria in cc.	0.2	0.2	0.2	0.2	0.2	0.2	
Covered with toluene and incubated at 37°C.							
Turbidity: Immediately	3	3	3	3	3	3	
Turbidity: After 6 hrs.	3	3−	3	3−	3	3−	
Turbidity: " 24 "	3	1	3	2−	3	1	

Separation, by Adsorption, of the Phage from the Agent Dissolving Dead Bacteria.

If freshly lysed cultures of staphylococci contain two active agents, as suggested by the experiment just described, it should be possible to separate them from each other also, by removing the phage from the solution through adsorption on live susceptible bacteria, and leaving in the solution only the agent dissolving dead bacteria.

Accordingly, cultures of Staphylococcus "G" were subjected to lysis by the appropriate bacteriophage, at 37°C. At the same time mass cultures of staphylococcus were grown on the surface of agar in Blake bottles. The next day bacteria

collected from the surface of three Blake bottles were washed by centrifugation, and to the solid mass of bacteria at the bottom of the centrifuge tube were added 20 cc. of lysed staphylococcus culture. At this time bacteria were suspended in the fluid above, by vigorous shaking, and the whole mixture was placed on ice for 2 hours, to allow for adsorption of the phage on bacteria. At the completion of a 2 hour period, the bacteria were thrown down by centrifugation at a high speed, for 1 hour. The centrifuge used for this purpose was supplied with a cooling device, so that lysis of bacteria was prevented during the experiment. A portion of the supernatant fluid was removed for examination of its bacteriophage content, as well as of its ability to dissolve dead bacteria, and to the remainder of the fluid was added another lot of bacteria collected from three Blake bottles. The mixture was shaken, placed on ice for 1 hour, and again bacteria were separated by centrifugation. The supernatant fluid was again tested as before. The results of these titrations are recorded in Protocol V.

As the results of this experiment indicate, it is possible to remove most of the bacteriophage proper by adsorption on bacteria, without affecting the power of the lysate to dissolve dead bacteria.

Dissolution of Dead Bacteria by Filtrates of Lysed Cultures in the Absence of Live Bacteria.

The last two experiments show that lysis of dead bacteria depends upon the presence in the freshly lysed cultures, in addition to the phage proper, of another active agent which does not go through the semipermeable membrane of collodion. This agent may conceivably be held back by the porcelain during filtration, and thus the fact that lytic filtrates, as usually prepared, do not cause the dissolution of dead bacteria becomes explainable. However, we found that the retention of this agent by the filter was complete and constant, while Gratia and Rhodes (8), report that the filtrates exhibit a certain amount of activity against old as well as against dead staphylococcus cultures.

Since the failure of lytic filtrates (bacteriophage) to dissolve old or dead bacteria constitutes, in our opinion, a characteristic which assumes fundamental importance in an attempt to understand the mechanism of transmissible lysis, we felt that it was necessary to determine beyond any doubt whether in the experiments of Gratia the active filtrates contained only the phage, or whether, under the conditions of his experiments, a certain amount of the second agent also had passed into the filtrate. Apart from the possibility that the

efficiency of the filters used by Gratia and ourselves might have been different, it seemed likely, that if large amounts of lysed cultures are filtered through a given filter, its efficiency may gradually decrease, and the substance, which at first is held back, may appear in the filtrate after continued filtration, thus possibly explaining the activity of his filtrates (26).

In order to test this possibility, a flask containing 1 liter of broth received a suitable amount of the suspension of the 18 hour agar growth of staphylococcus and of bacteriophage. The resulting mixture was distributed equally into three smaller flasks and incubated at 37°C. for 2, 4, and 6 hours respectively. At the end of 2 hours' incubation, one of the flasks was taken out and immediately subjected to fractional filtration through a new Berkefeld V candle, under pressure of 60 mm. of mercury as follows: At first 50 cc. of liquid were removed from the flask and filtered. The filtrate was collected into a sterile receptacle. Then a second 50 cc. portion of the contents of the flask was filtered through the same candle and the filtrate collected into a second receptacle. Then followed a third fraction of 50 cc. and so on—five fractions in all being employed. At this point the filter candle was discarded and the five fractions of the filtrate were immediately subjected to examination for sterility,[3] phage content, and for their power to cause lysis of dead staphylococcus, as indicated in Protocol VI, Section 1 A. At the proper intervals the contents of the other two flasks were similarly filtered, each through its own new filter candle, and the fractional filtrates thus obtained were examined, as indicated in Sections I B and I C of Protocol VI.

As another possibility which could explain discrepancies in the results it seemed to us of interest to inquire also into the rate of deterioration suffered by the agent responsible for the lysis of dead bacteria, under the influence of heat and preservation. In order to elicit the rôle of these factors, all the fractional filtrates (immediately after the removal of a small fraction of each for various tests, as shown in Sections I A, B, and C of the protocol) were divided into two portions each.

One portion of each filtrate was subjected to heating in sealed ampoules and submerged under water at 56°C. for 30 minutes. At the end of this time the tubes were removed from the water bath, cooled quickly in cold water, and the contents were tested for phage content and for their ability to cause the lysis of dead staphylococcus, as indicated in Sections II A, B, and C of Protocol VI.

[3] This control is essential, for if the filtrate contains live bacteria, the subsequent lysis of dead bacteria, if it occurs, cannot be attributed directly to passage of the active agent, but may be due to the lysis of live bacteria.

Protocol VI.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Culture of Staphylococcus during Progress of Lysis.

Duration of preliminary incubation.....	A (2 hrs.)											B (4 hrs.)												C (6 hrs.)											
Amount filtered in cc.....	Control	50		50		50		50		50		Control	50		50		50		50		50			Control	50		50		50		50		50		
Filtrate in cc.....	0	9	1	9	1	9	1	9	1	9	1	0	9	1	9	1	9	1	9	1	9	1		0	9	1	9	1	9	1	9	1	9	1	
Sterile broth in cc.....	9	0	9	0	9	0	9	0	9	0	9	9	0	9	0	9	0	9	0	9	0	9		9	0	9	0	9	0	9	0	9	0	9	
Dead staphylococcus suspension in cc.....	1	1	0	1	0	1	0	1	0	1	0	1	1	0	1	0	1	0	1	0	1	0		1	1	0	1	0	1	0	1	0	1	0	

Incubated at 37°C.

	Phage titer in cc.....		1 × 10^{-6}								1 × 10^{-6}			1 × 10^{-6}								1 × 10^{-6}								1 × 10^{-6}			
I	Turbidity { Immediately..	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0
	Turbidity { After 24 hrs...	4	4	0	4	0	4	0	3	0	2	0	4	4	0	4	0	3	0	2	0	2	0	4	4	0	2	0	1	0	1−	0	1−	0

Portions of each of the fractional filtrates were heated for 30 min. at 56°C. Mixtures were prepared exactly as above with broth and dead bacteria and incubated at 37°C.

	Phage titer in cc.....		1 × 10^{-6}								1 × 10^{-6}			1 × 10^{-6}								1 × 10^{-6}											
II	Turbidity { Immediately..	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0
	Turbidity { After 24 hrs..	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0

Portions of each of the fractional filtrates were kept at room temperature for 8 days. At the end of this time mixtures were made exactly as above with broth and dead bacteria, and incubated at 37°C.

	Phage titer in cc.....		1 × 10^{-6}								1 × 10^{-6}			1 × 10^{-6}								1 × 10^{-6}											
III	Turbidity { Immediately..	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0
	Turbidity { After 24 hrs...	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0	4	4	0	4	0	4	0	4	0	4	0

The remaining portion of fractional filtrates was allowed to stand in the room at ±25°C. for 8 days. After this period they were subjected to the test outlined in Sections III, A, B, and C of Protocol VI.

This experiment explains the discrepancy. It appears that when the filter is new and only a small amount of lysed cultures is filtered, only the phage appears in the filtrate, but as more and more of the solution is forced through the same candle, it becomes less selective and allows the passage of the second agent which is capable of causing dissolution of dead bacteria.[4] This is illustrated in the protocol by a diminution of the turbidity of bacterial suspensions in the tubes containing the filtrate, as compared with the turbidity of the controls. In the case in which the lysis in the original mixture was allowed to proceed only 2 hours before filtration (Protocol VI, Section I A), this agent appeared late—only after the passage of the fourth fraction—but as its concentration in the solution increased with the progress of lysis of live bacteria, it appeared in the filtrate sooner and in greater concentration, so that in the last section of the experiment (C) traces of the agent were already present in the second fractional filtrate, and in the fourth and fifth its concentration was sufficient to destroy almost all dead bacteria, as shown by the decrease of turbidity from 4 to 1. Comparison of the results in Sections I, II, and III, on the other hand, indicates that the agent lytic for dead bacteria is present only in freshly prepared filtrates of lysed cultures. If these filtrates are allowed to stand or are subjected to heating, this agent undergoes destruction, while the phage is still present in the solution and is unaffected.[5]

[4] Occasionally, on continued fractional filtration, not only the second active agent but also bacteria appeared in the filtrate, as was indicated by the sterility controls. Such experiments were discarded and repeated, until sterile filtrates were obtained. These findings are of especial interest in connection with the statements in the literature, in which the appearance of growth in the filtrates of lysed cultures is attributed to the existence of a filterable stage in the life cycle of bacteria (13, 14, 26).

[5] While in the experiment recorded in Protocol VI the agent causing the dissolution of dead bacteria seems to be completely destroyed on standing or on exposure to heat at 56°C. for 30 minutes, this is not always the case. Repeating these experiments and employing suspensions of dead bacteria of varying density, we found that this destruction is usually very marked but not complete—with

Failure of Filtrates of a Live Resistant Variant, Grown in the Presence of Bacteriophage, to Induce Lysis of Dead Staphylococcus.

We have shown in the preceding experiments that bacteriophage has no direct lytic action on dead bacteria. Apparently by causing the lysis of susceptible live staphylococcus, it merely sets free a lytic agent preexisting in the bacteria themselves.

If this inference is correct, the incubation of bacteriophage with a live resistant variant instead of with a susceptible staphylococcus should yield a filtrate that will not lyse dead bacteria. Such an experiment was performed with a resistant variant isolated from the susceptible strain of Staphylococcus "G" used previously. It was found that when the experiment was carried out in a manner identical with that shown in Protocol VI, no dissolution of the dead bacteria occurred. On the other hand, dead resistant bacteria were as susceptible to the lytic action of the agent produced by the live susceptible bacteria as were the dead susceptible organisms themselves.

Evidently the failure of the phage to cause the lysis of resistant staphylococcus precluded the liberation of the lytic agent from the latter. However, when susceptible live bacteria were used, the lytic agent was set free and was able to dissolve either the susceptible or resistant dead staphylococci.

In general, the results of the preceding experiment confirm our earlier observations, namely, that the agent responsible for the transmissible lysis of live bacteria is different and independent from that which causes the lysis of dead bacteria. Moreover, the fact that the latter ferment-like agent appears comparatively late during the lysis of live bacteria, that it is thermolabile, and is inactivated on standing,[6] that it is specific in its action, that it does not go through the collodion membrane, that it passes the filters only with difficulty, and causes dissolution of dead bacteria, suggests the possibility that it may be identical with the autolytic endoenzyme which is set free during the lysis of live bacteria.

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very light suspensions of bacteria some residual activity can still be detected, when heavier suspensions of bacteria show no apparent presence of the lytic agent, as will be shown later (Protocol IX).

[6] This inactivation is probably due to its combination with the products of its own activity.

Analogy between the Agent Dissolving Dead Bacteria and the Autolytic Enzyme.

In order to determine whether this ferment-like agent, destroying dead staphylococcus, can be identified with the autolytic endoenzyme of this organism, we repeated some of the experiments described above, using the enzyme obtained from cultures of staphylococcus grown in the absence of phage.

Protocol VII.

Presence of Specific Bacteriolytic Agent in the Young Cultures of Staphylococcus.

	Dead staphylococci		Dead *B. coli*		Dead *B. pestis caviæ*		Dead *B. dysenteriæ*	
Supernatant fluid from staphylococcus culture in cc.	5	0	5	0	5	0	5	0
Broth in cc.	0	5	0	5	0	5	0	5
Suspension of dead bacteria in cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Covered with toluene and incubated at 37°C.								
Turbidity: Immediately	5	5	5	5	5	5	5	5
Turbidity: After 5 hrs.*	2	5	5	5	5	5	5	5

* The contents of these tubes were tested for the presence of phage after the completion of the experiment in order to exclude the possibility of lysis having occurred as the result of accidental introduction of bacteriophage.

The culture of staphylococcus was grown in broth for 18 hours, either aerobically or anaerobically, the bacteria were removed by centrifuging for 1 hour at high speed, and 5 cc. of the supernatant fluid were placed in a tube, to which was added a suspension of dead staphylococcus. Another tube containing 5 cc. of sterile broth received a similar amount of the same suspension of dead bacteria. The initial turbidity of the resulting suspensions was recorded by comparison with the standard turbidity scale. Chloroform or toluene was then added to the contents of each tube as a preservative, and the tubes were incubated at 37°C. At the expiration of 5 hours turbidity was again compared with the standard scale and changes recorded, as illustrated in Protocol VII.

In a series of experiments identical with the one just described, suspensions of dead *B. coli, B. pestis caviæ* and *B. dysenteriæ* (Shiga) were subjected to the action of the supernatant fluid from the 18 hour growth of staphylococcus, in order to determine whether the action on dead bacteria was specific, and the results were similarly recorded in terms of turbidity of

the suspensions, immediately after the addition of dead bacteria, and after 5 hours of incubation at 37°C. (Protocol VII).

Thus, in the 18 hour culture of staphylococcus grown in broth in the absence of bacteriophage there was present a specific bacteriolytic agent (presumably enzyme) active against dead staphylococcus. In order to identify more closely this bacteriolytic agent with that which was found to be present in cultures of staphylococcus undergoing lysis by the bacteriophage, we repeated the fractional filtration experiment (see Protocol VI), employing the supernatant fluid of a broth culture of staphylococcus instead of the fluid resulting from the progressive lysis of staphylococcus by the bacteriophage (Protocol VIII). The experiment was carried out exactly as before (Protocol VI) and a detailed description of the procedure has therefore been omitted.

As will be seen, this experiment shows that even after 6 hours of growth the cultures of staphylococcus do not contain enough enzyme in solution for it to be demonstrable by the method used. However, after 18 hours of growth there is a measurable amount of the enzyme present. If these findings are compared with corresponding results in the preceding experiment, it will be observed that in the presence of bacteriophage the enzyme-like substance appears earlier and is present in considerably greater concentration (Protocol VI, Section I), since even the filtrate of 2 hours' growth contains the active agent. This difference might have been expected, since in the presence of phage the rate of growth is more rapid, and also because a number of bacteria are undergoing early lysis, thus setting free the enzyme, whereas in the absence of the phage the enzyme appears in solution, coincident with the late autolysis of bacteria. Moreover, in both cases the first fractional filtrates of the cultures presumably containing the enzyme do not show any activity, and it is only on repeated fractional filtration that the activity becomes demonstrable (compare Protocol VI, Section I A with Protocol VIII, D). Similarly, it was found that exposing to heat the filtrates containing the enzyme, or keeping them for days in the laboratory results in a gradual destruction of the active agent in a manner entirely analogous to that observed in the case of the filtrates of lysed cultures of staphylococcus (Protocol VI, Sections II and III). Here again deterioration of the enzyme

Protocol VIII.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Broth Culture of Staphylococcus.

Duration of preliminary incubation.....	A (2 hrs.)					B (4 hrs.)					C (6 hrs.)					D (18 hrs.)				
Amount filtered in cc.....	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50
Amount of filtrate in cc.....	0	9	9	9	9	0	9	9	9	9	0	9	9	9	9	0	9	9	9	9
Sterile broth in cc.....	9	0	0	0	0	9	0	0	0	0	9	0	0	0	0	9	0	0	0	0
Dead staphylococcus suspension in cc..	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Incubated at 37°C. and turbidity determined at intervals.																				
Immediately.....	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
After 2 hrs.....	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 4 "	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 24 "	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2−	2−
" 48 "	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1	1−

appeared complete when its activity was tested with heavy suspensions of bacteria, but when lighter suspensions were used, destruction of the enzyme under the conditions of the experiment was found to be incomplete as will be seen in Protocol IX.

So far as we have inquired into the behavior of the unknown agent appearing in cultures of staphylococcus during lysis under the influence of the bacteriophage, and responsible for the dissolution of dead bacteria, it appears in all respects analogous to the autolytic enzyme which can be obtained from older cultures of staphylococcus grown without the phage.

Protocol IX.

The Effect of Density of Bacterial Suspension on the Outcome of the Test of Activity of the Enzyme.

Filtrate of lysed culture kept for 8 days at room temperature in cc.	5			5			5		
Filtrate of 18 hr. old culture of staphylococcus kept for 8 days at room temperature in cc.		5			5			5	
Sterile broth in cc.			5			5			5
Suspension of dead staphylococcus in cc.	0.05	0.05	0.05	0.1	0.1	0.1	0.15	0.15	0.15
Incubated at 37°C.									
Turbidity: Immediately	2	2	2	4	4	4	6	6	6
Turbidity: After 4 hrs.	2	2	2	4	4	4	6	6	6
Turbidity: " 24 "	1−	2−	2	3	4	4	6	6	6
Turbidity: " 48 "	1−	1	2	2	3	4	6−	6	6

The Effect of the Lysis of Dead Bacteria on the Titer of the Phage.

Our experiments show, we believe, conclusively that the enzyme-like agent is distinct from phage, and its early appearance in the culture is merely incidental to the lysis of living bacteria and in no way connected with the activity of the bacteriophage itself. However, in view of the fact that Gratia and Rhodes (8) report probable regeneration of phage during the lysis of dead bacteria, we investigated this point.

For this purpose, live staphylococci and a corresponding phage were introduced into a flask containing 200 cc. of sterile broth, and incubated at 37°C. for 4 hours. Up to that time the lysis had taken place only partially, and the titer of phage had reached 1×10^{-6} cc., as illustrated in Protocol X. The bulk of the contents of the flask, with the exception of 10 cc., was filtered through a sterile candle in order to thoroughly saturate it, and the filtrate was discarded. The last 10 cc. of the culture were then filtered through the same candle and to this filtrate—presumably containing both the phage and the agent active for dead bacteria—was added a suspension of dead bacteria. The turbidity of the resulting mixture was estimated, a sample was taken out for the immediate titration of the phage content, and the tube was placed in the incubator. After 24 hours of incubation the turbidity and phage content were reestimated.

Protocol X.

Effect of Dead Bacteria on the Phage Titer.

Filtrate in cc	10	0	10
Broth in cc	0	10	0
Suspension of dead bacteria in cc	0.2	0.2	0

Incubated at 37°C.

	Turbidity	Phage titer	Turbidity	Phage titer	Phage titer
		cc.			*cc.*
Immediately	4	10^{-6}	4	0	10^{-6}
After 24 hrs.	3—	10^{-1}	4	0	10^{-6}

The experiment shows that during the partial lysis of dead bacteria there was no increase in the phage. On the contrary, the bulk of the phage disappeared from the solution during the incubation, probably having been adsorbed on dead bacteria.

DISCUSSION.

According to the original conception of d'Hérelle (15), the clearing of bacterial suspensions in the presence of bacteriophage is the result of gradual swelling and eventual bursting of bacteria, due to the accumulation within them of multiplying parasites (*Bacteriophagum intestinale*). Following this bursting, the young parasites are set free in increased numbers to invade other bacteria, and the débris of ruptured bacterial cells is dissolved by the action of lysin secreted by the parasite (16).

Hence, the increase of the concentration of bacteriophage in solution is preceded by the bursting of bacteria, and the destruction of the latter is considered essential for the regeneration of the phage. However, it has been shown repeatedly that the phage titer of a culture shows an increase before the onset of actual lysis of bacteria, and indeed, under certain conditions, it may reach very high concentration without any lysis of susceptible bacteria (17–21). Thus, while lysis of bacteria is the most striking feature of the d'Hérelle phenomenon, it evidently plays no part in the production or regeneration of the bacteriophage, and when it occurs it is secondary to more essential, though obscure changes in bacterial cells which are accompanied by an increase of phage titer. Just what the nature of the process is by which bacteria undergo complete dissolution in the phenomenon of d'Hérelle has not been definitely established. We have shown (22) that there is no valid evidence of the existence of "lysin," as postulated by d'Hérelle, as a secretion of the ultraparasite (16). Moreover, if bacterial débris is dissolved by an enzyme-like lysin secreted by the *Bacteriophagum intestinale*, one would certainly expect that such a lysin would also dissolve dead bacteria, whereas all the experimental data presented thus far indicate that only live and actively growing bacteria are subject to lysis by the phage.

The findings of Twort seemed to us to have offered a means of solving this problem. We have been able to confirm his observations that dead bacteria remain unaffected by the bacteriophage alone, but that they undergo lysis if, in addition to the bacteriophage, homologous live bacteria are present. We have shown in the experiments reported in this paper that bacteriophage itself takes no part in the dissolution of dead bacteria, but acts merely as an incitant for certain changes occurring in live bacteria and leading to their eventual lysis. The dissolution of the dead bacteria takes place at the expense of a lytic enzyme, set free as the result of lysis of the live bacteria.

These findings, taken with our observations on the viscosity of bacterial suspensions in the presence of bacteriophage (23), lead us to infer that the determining factor in the failure of bacteriophage to bring about dissolution of resistant or old bacteria is to be looked for in the failure of these bacteria to swell under the influence of the phage. Apparently the swelling itself (by dilution of intracellular

contents?), or the bursting of live bacteria as the result of the intake of water, is followed by the dissociation of the intracellular enzyme-antienzyme complex (24), with consequent activation of the autolytic enzyme, which attacks not only the débris of ruptured young bacteria, but if present at the same time, also the dead bacteria.

SUMMARY AND CONCLUSIONS.

We have been able to confirm the observations of Twort as well as of Gratia, that dead staphylococcus may undergo lysis if, in addition to a suitable bacteriophage, there is also present live staphylococcus. Moreover, we have endeavored to ascertain the mechanism of this phenomenon and have found that in order to elicit it it is necessary to control the numbers of live and dead bacteria in the mixture. An excess of dead bacteria interferes with lysis by adsorbing the bacteriophage before it has the opportunity to initiate necessary changes in the live bacteria, so that all lysis is prevented. The phenomenon is specific, that is, the lysis of live bacteria is accompanied by lysis of dead bacteria of the same species only. Lysis of dead bacteria occurs best with staphylococcus, an organism which easily undergoes spontaneous autolysis under appropriate conditions. In the case of *B. coli* or *B. dysenteriæ* the lysis of the dead bacteria is uncertain. Dead bacteria need not be present in the mixture at the beginning of the experiment; they will be dissolved if added any time before, during, or after the completion of lysis of live bacteria.

If the test is performed so that a suitable semipermeable membrane is interposed between the dead and live bacteria, the dead bacteria are not dissolved, in spite of the lysis of live bacteria on the other side of the membrane. The agent determining the lysis of dead bacteria is not diffusible, while the principle initiating the lysis of live bacteria diffuses freely and is demonstrably present on both sides of the membrane. The complete independence of the agent causing dissolution of dead bacteria from bacteriophage can also be shown by separating the two agents by means of filtration, or by adsorption on bacteria.

The ferment-like substance responsible for the lysis of dead bacteria is different from the bacteriophage. It is not diffusible through collodion, it is easily adsorbed on clay filters, it is heat-labile, and is inactivated on standing.

An agent possessing identical properties was found in cultures of staphylococcus undergoing spontaneous autolysis in the absence of bacteriophage, but in this instance the agent appeared in the filtrates considerably later than it did when phage was present.

BIBLIOGRAPHY.

1. Jötten, K. W., *Klin. Woch.*, 1922, i, 2181.
2. Wollman, E., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 3.
3. d'Hérelle, F., The bacteriophage. Its rôle in immunity, translation by Smith, G. H., Baltimore, 1922.
4. Zinsser, H., Parker, J. T., and Kuttner, A., *Proc. Soc. Exp. Biol. and Med.*, 1920, xviii, 49; Kuttner, A., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 158, 222.
5. Seiffert, W., *Z. Immunitätsforsch., Orig.*, 1923, xxxviii, 292.
6. Otto, R., and Munter, H., *Z. Hyg. u. Infectionskrankh.*, 1923, c, 402.
7. Doerr, R., and Grüninger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 209.
8. Gratia, A., and Rhodes, B., *Compt. rend. Soc. biol.*, 1923, lxxxix, 1171.
9. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1926, xciv, 242.
10. Gratia, A., and Rhodes, B., *Compt. rend. Soc. biol.*, 1924, xc, 640.
11. Twort, F. W., *Lancet*, 1925, ccix, 642.
12. Bronfenbrenner, J., and Muckenfuss, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 633.
13. Hauduroy, P., *Compt. rend. Soc. biol.*, 1926, xciv, 661.
14. Fejgin, B., *Compt. rend. Soc. biol.*, 1925, xcii, 1528.
15. d'Hérelle, F., The bacteriophage. Its rôle in immunity, translation by Smith, G. H., Baltimore, 1922, 63.
16. d'Hérelle, F., The bacteriophage. Its rôle in immunity, translation by Smith, G. H., Baltimore, 1922, 123.
17. Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.
18. Otto, R., and Munter, H., *Z. Hyg. u. Infectionskrankh.*, 1923, c, 402.
19. Brutsaert, *Arch. méd. Belges*, 1924, lxxvii, 839.
20. da Costa Cruz, J., *Compt. rend. Soc. biol.*, 1924, xci, 840.
21. Matsumoto, T., *Centr. Bakt., 1. Abt., Orig.*, 1924, xci, 413.
22. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 419.
23. Bronfenbrenner, J., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 635.
24. Northrop, J. H., *J. Gen. Physiol.*, 1922, iv, 245; Hussey, R. G., and Northrop, J. H., *J. Gen. Physiol.*, 1923, v, 335.
25. Bronfenbrenner, J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483.
26. Bronfenbrenner, J., and Muckenfuss, R., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 371.

BACTERIUM LEPISEPTICUM INFECTION.

Its Mode of Spread and Control.

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(Received for publication, January 14, 1927.)

The experiments outlined in this paper were designed to throw some light on the natural mode of spread of *Bact. lepisepticum* infection and to determine a method for its control. The technique employed is based upon the results of a number of previous observations of the spontaneous disease as it occurs in rabbits (1,*a*, 2) and upon measurements of certain properties of host and microbe under limited but rigidly controlled circumstances (1,*b*). It was hoped, therefore, that by eliminating certain variables and submitting certain conceptions to a critical test under natural conditions, it would be possible to ascertain accurately the factors underlying the various phenomena observed.

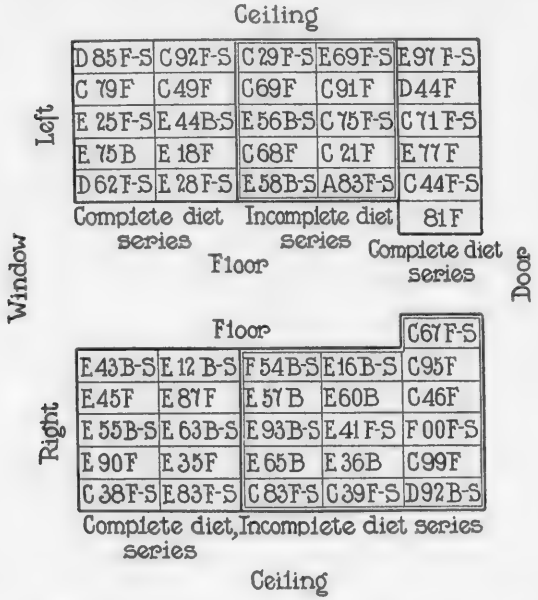
The test may be divided conveniently into two parts; first, an effort to keep a certain population of rabbits entirely free from *Bact. lepisepticum* infection; second, a study of the behavior of another population of rabbits exposed to the disease in a random and natural manner. To carry out the first part of the experiment, a specially selected group of animals was chosen and placed in a well isolated room, under the care of an experienced attendant; for the second test, the animals were also chosen with care and placed in an isolated room, but were exposed to the risk of infection by a general rabbit caretaker. The details of each test and the results obtained follow.

Spread of Spontaneous Bact. lepisepticum Infection among a Specially Controlled Group of Rabbits.

Materials.—52 rabbits, 3 months old, weighing 800 to 1000 gm. each, were chosen for this experiment. Thirty-six came from a nearby farm which was reported free from cases of clinical snuffles; sixteen were taken from the Rockefeller

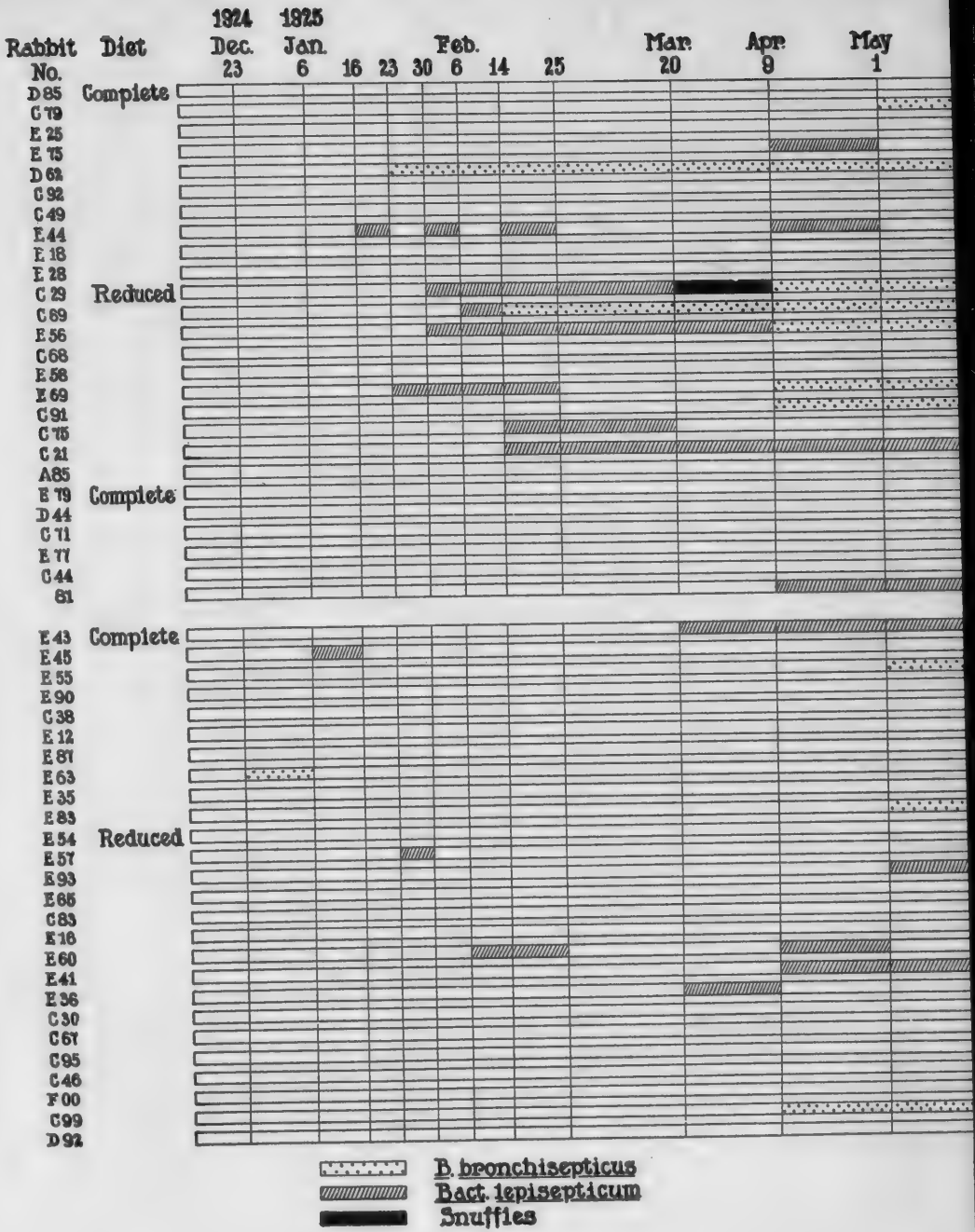
Institute breeding room described below. Animals in this breeding room had been entirely free from *Bact. lepisepticum* carriers for 1 year. Hence it was inferred that the young animals employed in this experiment had never been exposed to *Bact. lepisepticum*. The nasal passages of the 52 animals were cultured three times to discover the presence of *Bact. lepisepticum* carriers. None was found.

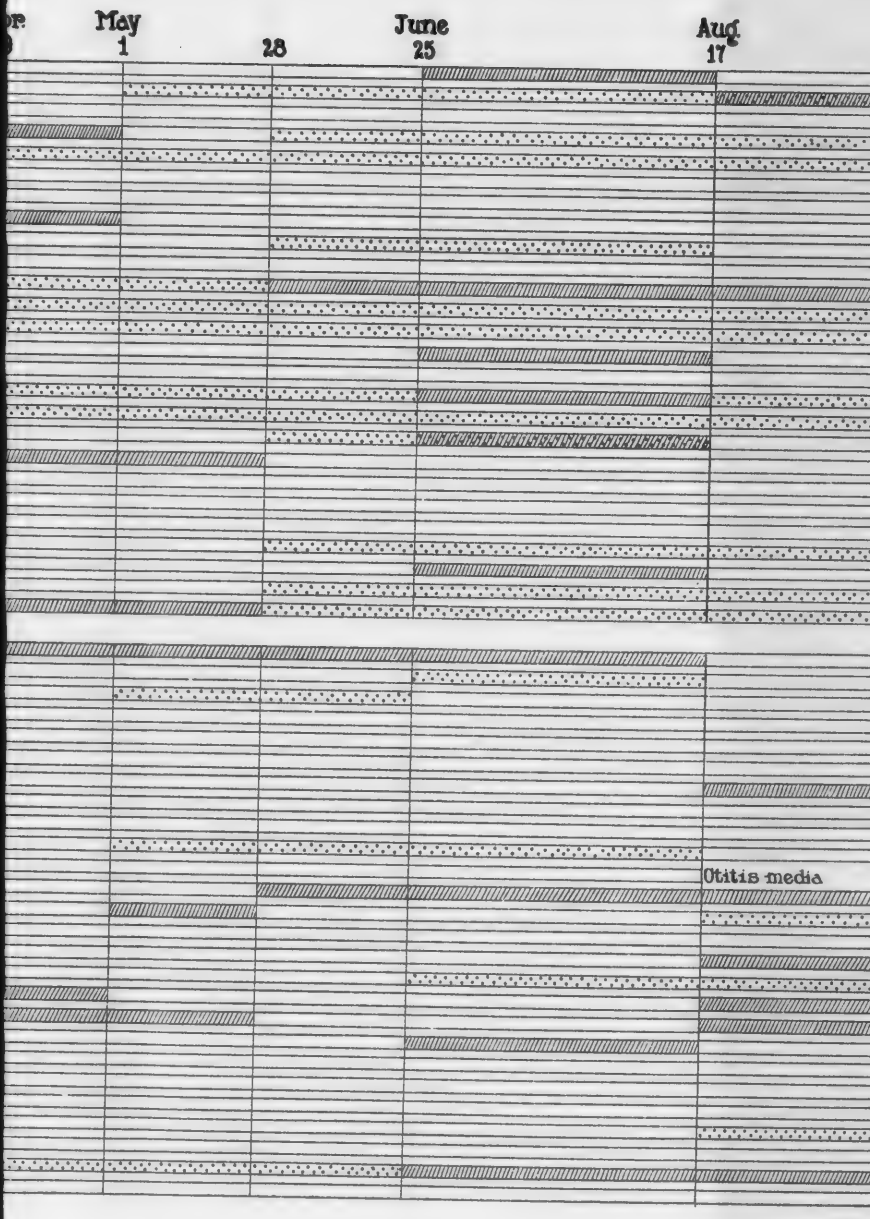
The rabbits were placed in separate cages measuring about 12 × 12 × 22 inches in dimensions, and distributed uniformly in four stacks of five cages, and one

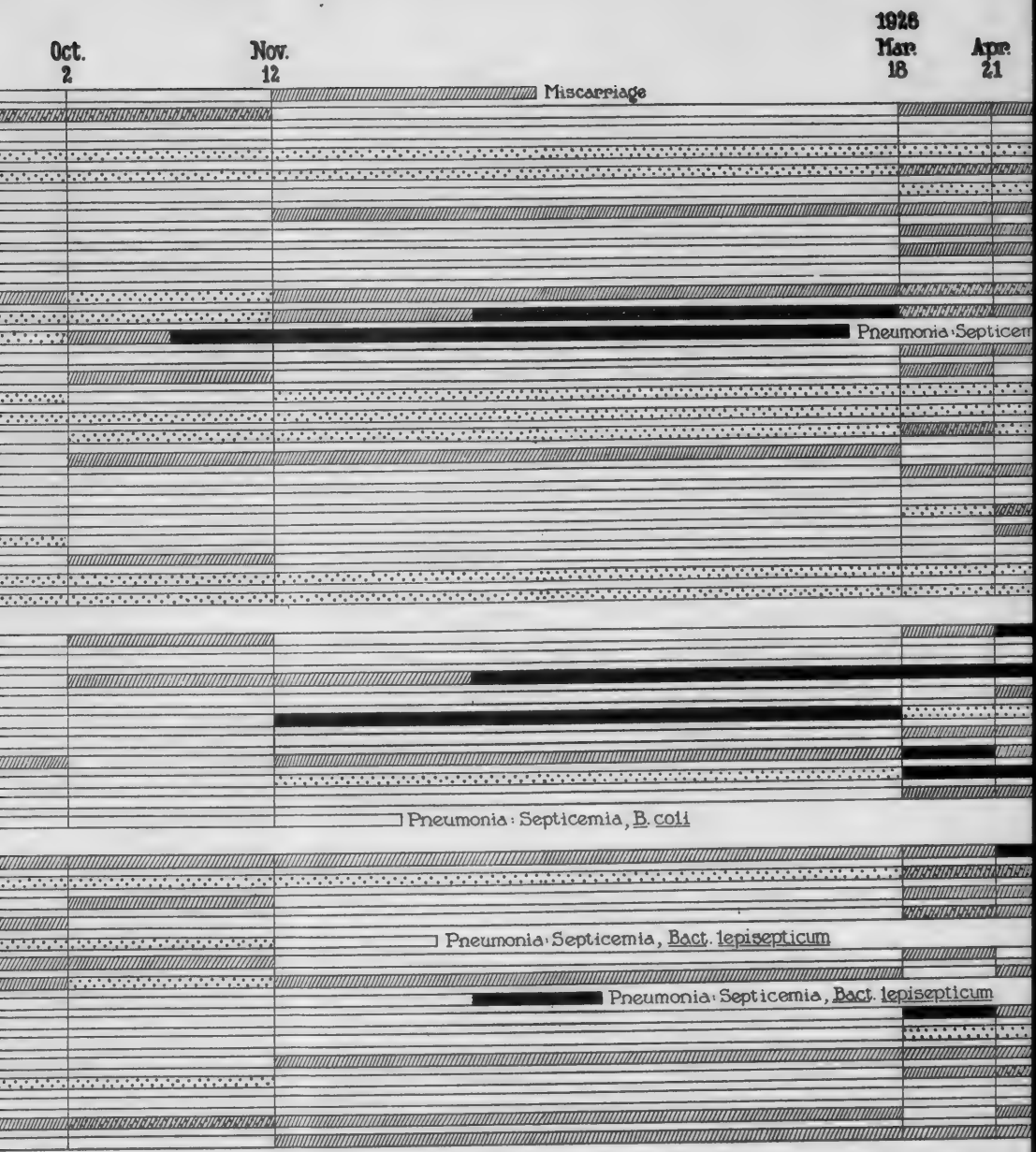


TEXT-FIG. 1. Plan of experimental room showing arrangement and location of animals. F following rabbit numbers = outside farm source; B = breeding room source; -S = depilation, October, 1925.

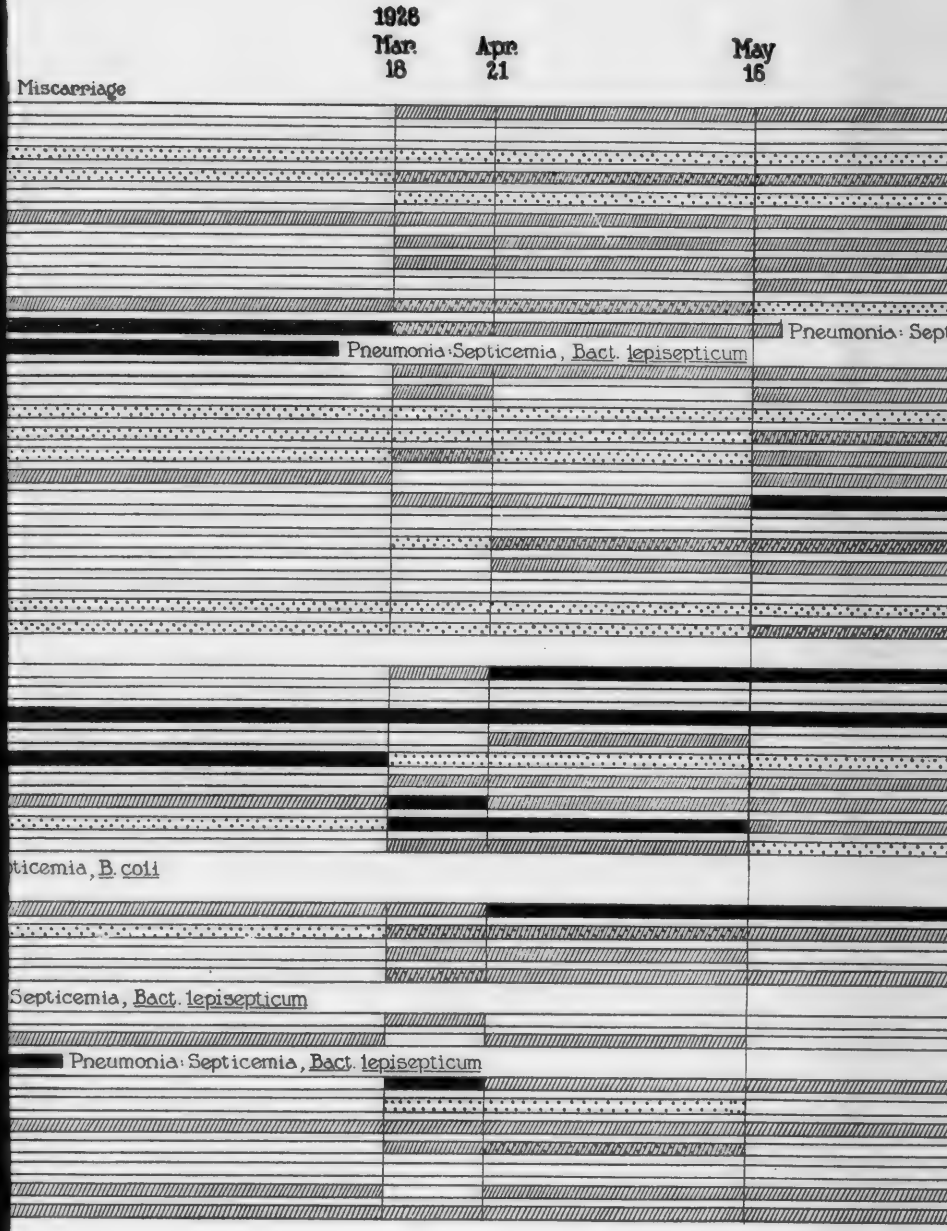
stack of six, on two sides of a room about 10 feet wide and 20 feet long. A window was located at one end of the room, a door at the other (Text-fig. 1). The temperature was maintained as nearly as possible at 68°, except during one definite interval described below. Two distinct diets were employed—a "complete" ration, consisting of oats, first quality hay, and fresh cabbage or carrot, plus 2 or 3 cc. of cod liver oil daily, and an "incomplete" diet of the oats and hay, plus water without any fresh vegetable or cod liver oil. The animals were cared for by attendants who were also in charge of several hundred other rabbits nearby, many of which suffered from snuffles and carried *Bact. lepisepticum*.


1924
1825
Rabbit No.
Diet
Dec. 23
Jan. 6
16
23
30
Feb. 6
14
25
Mar. 20
Apr. 9
May 1
D 85
C 79
E 25
E 75
D 62
C 92
C 49
E 44
E 18
E 28
C 29
C 69
E 56
C 68
E 58
E 69
C 91
C 75
C 21
A 85
E 79
D 44
C 71
E 77
C 44
61
E 43
E 45
E 55
E 90
C 38
E 12
E 87
E 63
E 35
E 83
E 54
E 57
E 93
E 65
C 83
E 16
E 60
E 41
E 36
C 30
C 67
C 95
C 46
F 00
C 99
D 92
Complete
Reduced
Complete
Complete
Reduced
B. bronchisepticus
Bact. lepisepticum
Snuffles

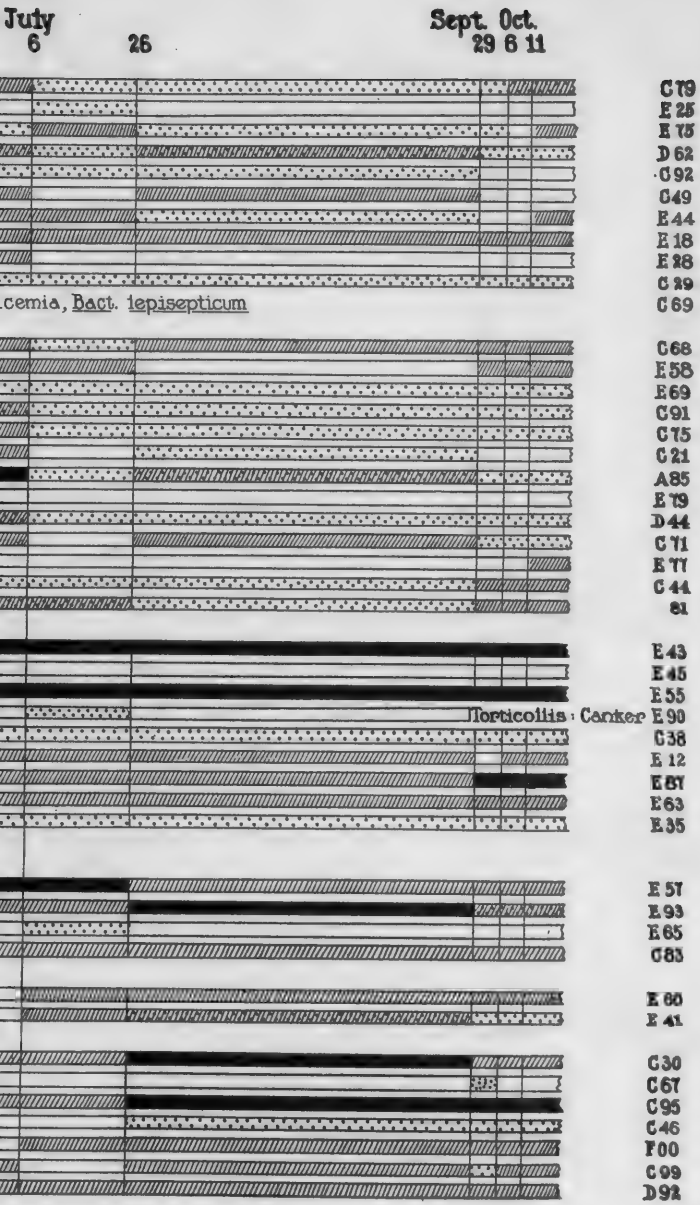

May
1
28
June
25
Aug.
17
Otitis media



ıc. 2. Results of clinical and bacteriological examinations of 52 rabbits.


1928
Mar.
18
Apr.
21
May
16
Miscarriage
Pneumonia·Septicemia, Bact. lepisepticum
Pneumonia: Sept
ticemia, B. coli
Septicemia, Bact. lepisepticum
Pneumonia·Septicemia, Bact. lepisepticum

abbits.


July
6
26
Sept. Oct.
29 6 11
C 79
E 25
E 75
D 62
C 92
C 49
E 44
E 18
E 28
C 29
cemia, Bact. iepisepticum
C 69
C 68
E 58
E 69
C 91
C 75
C 21
A 85
E 79
D 44
C 71
E 77
C 44
81
E 43
E 45
E 55
Torticollis · Canker
E 90
C 38
E 12
E 87
E 63
E 35
E 57
E 93
E 65
C 83
E 60
E 41
C 30
C 67
C 95
C 46
F 00
C 99
D 92

Method.—The group of 52 animals was then submitted to the chances of random infection through the vehicle of the caretakers, the bedding, food, insects, etc. Throughout a period of 2 years as many factors as possible were controlled and analyzed. In order to do this with so small a group, every precaution was taken to keep the experimental variables balanced. Thus, the animals from the breeding room were scattered uniformly among those from the farm. The diet groups were likewise evenly distributed so as to include both series of animals and both sides of the room (Text-fig. 1). The population consisted therefore of a group of animals among which was operating a definite number of controlled and balanced variables.

The animals were kept under strict observation. Notes were made of their general health, the presence or absence of snuffles, and other pathological processes. Nasal cultures were taken at frequent intervals (1, *c*) to determine *Bact. lepisepticum* carriers. All animals dying during this period were autopsied carefully.

After the experiment had been in progress for about 10 months, it was decided to modify atmospheric conditions in the room by arbitrarily altering the temperature and humidity. This was done by turning on the steam heat to full strength in the morning, shutting the doors and windows, and turning on the hot water. The room became filled rapidly with water vapor and reached a temperature of about 90°. At night the heat and hot water were shut off and the window opened wide. This caused the temperature of the room to drop to that prevailing out of doors. These extreme measures were employed from October 22, 1925, until February 4, 1926. At the same time the backs of the animals on the top, middle, and bottom rows on each side of the room were depilated from neck to buttocks by means of sodium sulfide (Text-fig. 1).

On December 1 the surviving animals were given intranasally a 16 hour blood broth culture of the virulent Rivers D strain of *Bact. lepisepticum* (1, *d*). And on December 18 to 23 the entire group was killed and autopsied. Cultures were taken from nasal passages, ears, and lungs.

Results.—The results of these observations are shown in Tables I and III and Text-fig. 2. They will be considered in detail with reference to (*a*) the total population, (*b*) the groups on either side of the room, (*c*) the groups from the farm and breeding room, (*d*) the diet groups, (*e*) seasonal factors, including temperature changes and exposure, and (*f*) individual differences in response on the part of the animals.

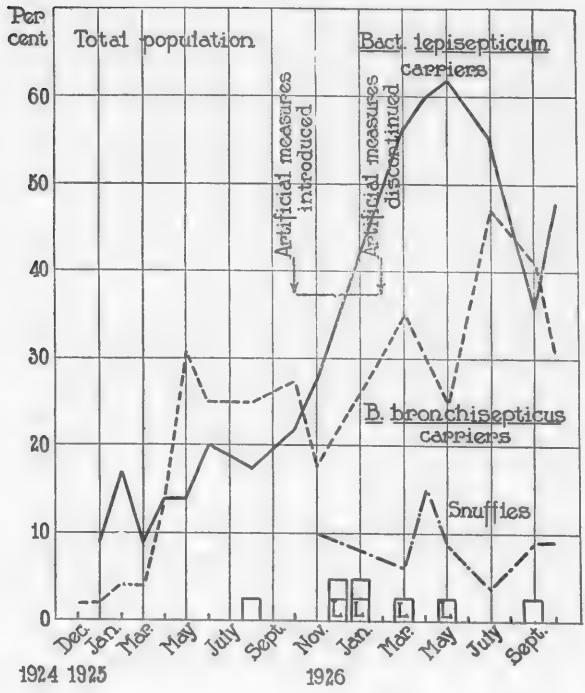
(*a*) *Total Population.*—The outstanding events referable to the total population are taken from Table I and Text-fig. 2, and charted in Text-fig. 3. *Bact. lepisepticum* carriers appeared in January, 1925, increased at a more or less uniform rate from 10 per cent to 22 per cent in October. *B. bronchisepticus* carriers rose from 4 per cent in March to 31 per cent in May, and fell to a level of about 25 per cent from

TABLE I.

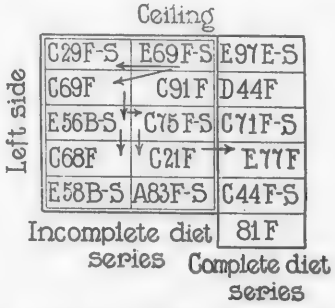
Summary of Findings on Experimental Group of 52 Rabbits.

Side	Diet	Condition	Dec., 1924	Jan., 1925	Feb.	Mar.	Apr.	May	June	Aug.	Oct.	Nov.	Dec.	Mar., 1926	Apr.	May	July	Sept.	Oct.
Left (26)	Complete (16)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1†	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Carriers *Bact. lep.*	0	1	1	0	3	1	2	1	2	2	—	5	7	9	7	3	7
		Carriers *B. bronch.*	0	1	1	1	1	7	7	6	5	4	—	6	6	6	9	6	4
	Incomplete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1‡	0	1‡	0	0	0
		Snuffles	0	0	0	1	0	0	0	0	0	1	2	0	0	1	0	0	0
		Carriers *Bact. lep.*	0	3	6	3	1	2	4	1	3	4	—	6	4	6	3	2	2
		Carriers *B. bronch.*	0	0	1	1	5	6	4	4	4	3	—	5	3	2	7	5	5
Right (26)	Complete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	1†	0	0	0	0	1*	0
		Snuffles	0	0	0	0	0	0	0	0	0	1	2	3	3	2	2	3	3
		Carriers *Bact. lep.*	0	1	0	1	1	1	1	1	2	3	—	6	7	5	5	4	6
		Carriers *B. bronch.*	1	0	0	0	0	2	2	0	0	1	—	1	1	2	4	3	2
	Incomplete (16)	Deaths	0	0	0	0	0	0	0	1*	0	0	2‡	0	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	1	1	1	1	3	1	1
		Carriers *Bact. lep.*	0	1	1	1	2	3	3	5	4	5	—	9	10	7	10	8	9
		Carriers *B. bronch.*	0	0	0	0	1	1	1	3	5	1	—	4	3	0	2	5	3

* Otitis media: *Bact. lepisepticum.* † Septicemia: *B. coli.* ‡ Pneumonia: septicemia: *Bact. lepisepticum.*



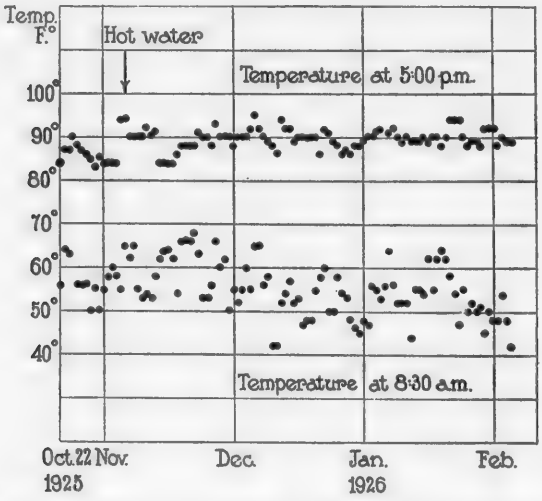
TEXT-FIG. 3. Reaction of the total population to *Bact. lepisepticum* infection.



TEXT-FIG. 4. Cage to cage spread of *Bact. lepisepticum* infection from a single focus, January 30, 1925, to June 25, 1925.

June to October. One death occurred in August. This animal showed bilateral otitis media.

During this period and throughout the 2 years of observation, a spot map was kept, showing the location and spread of *Bact. lepisepticum* and *B. bronchisepticus* carriers. The early months showed random and scattered foci of *Bact. lepisepticum* infection with a tendency toward local spread. One such focus is charted in Text-fig. 4. The diagram includes the "incomplete" diet series on the left side of



TEXT-FIG. 5. Daily temperature fluctuations in experimental rabbit room.

the room, and six adjacent animals of the "complete" diet series (see Text-fig. 1). The date of appearance of *Bact. lepisepticum* among these animals, recorded in Text-fig. 2, is shown here chronologically by means of arrows.

Apparently the infection started with Rabbit E 69 F-S, and extended to the two adjacent cages, C 29 F-S and C 69 F. From this latter it progressed to E 56 B-S and from there to the three contiguous animals, C 75 F-S, C 68 F, and C 21 F. Finally, it appeared in E 77 F. Evidently, therefore, direct extension of the infection did occur. This possibility appeared the more probable when it was learned that the

attendant always fed the animals in vertical rows, from ceiling to floor. It must be remembered, however, that new foci were appearing throughout the population, and that the attendant was continually passing from the animals in this room to other badly infected stock nearby. Hence, two sources of infection were continually in operation; one, a local spread from established foci in the population, and second, an importation of organisms from without by the attendant.

On October 22, 1925, the artificial temperature fluctuations described above were begun. These procedures continued until February 4, 1926. Daily temperature fluctuations are indicated in Text-fig. 5, in which the 8.30 o'clock morning readings and 5.00 o'clock afternoon readings are recorded. Twice during every 24 hours, therefore, a fluctuation of approximately 35° occurred. Furthermore, on November 6, the hair was removed from the backs of one-half of the entire population (see Text-fig. 1).

These procedures were followed by a striking increase in the percentage of *Bact. lepisepticum* carriers, the appearance of acute and chronic snuffles, and the death of six animals within 7 months. *Bact. lepisepticum* carriers rose to 56 per cent in March, 60 per cent in April, and 62 per cent in May; snuffles appeared and became chronic among about 10 per cent of the population; two animals died in December, two in January, and one in March and May, respectively. Four of the fatalities were due to *Bact. lepisepticum* septicemia or pneumonia. No definite increase of *B. bronchisepticus* carriers was noted during this time. This marked increase in the spread of *Bact. lepisepticum* infection, accompanied by clinical snuffles and a definite mortality, was associated so conspicuously with cold weather and the experimental temperature fluctuations that a causal relation was inferred.

From May to September there was a sharp decline in the percentage of *Bact. lepisepticum* carriers, a decline in the number of cases of clinical snuffles, and in mortality. However, the number of *B. bronchisepticus* carriers increased.

(*b*) *Left and Right Sides of the Room.*—When the prevalence of *Bact. lepisepticum* infection on the right and left sides of the room is compared (Table I), the same general phenomena are noted. For the first 6 months carriers seemed more abundant on the left side, but during the winter of 1926 the rise was uniform. During the summer

of 1926, however, the carrier rate fell on the left side more rapidly than on the right. Chronic snuffles was more prevalent on the right side, and deaths were about equally distributed.

(*c*) *Breeding Room and Farm Groups.*—The animals from the breeding room appeared to be somewhat more susceptible than those from the farm (Table II). A summary was made at the end of the experiment of the number of deaths, the number of cases of chronic snuffles, of chronic and occasional carriers of *Bact. lepisepticum*, and of animals free of the infection. 25 per cent of the breeding room animals died, as compared with 14 per cent of the farm animals; 31 per cent of the former showed chronic snuffles, 14 per cent of the latter. About the same relative numbers of each group were chronic carriers, but none

TABLE II.

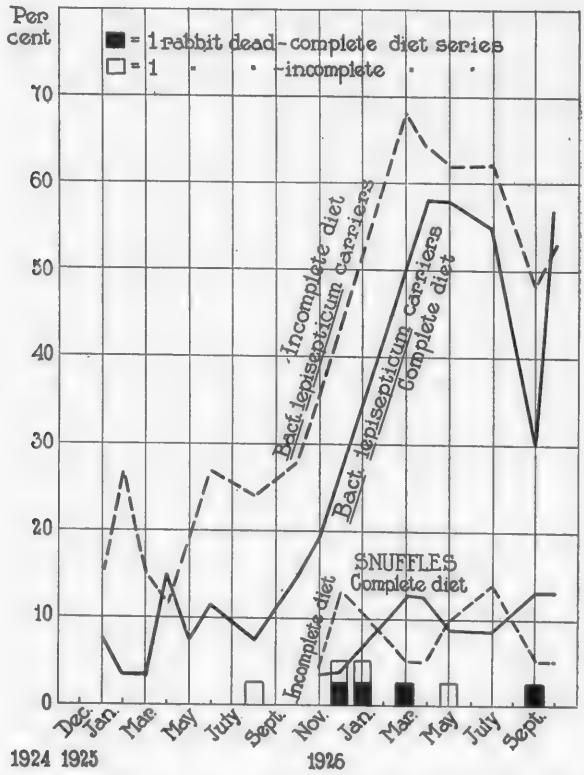
Comparison of Breeding Room and Farm Rabbits and Depilated and Non-Depilated Rabbits.

Group	Died		Chronic snuffles		Chronic carriers		Intermittent carriers		Free of *Bact. lepisepticum*	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Breeding room (16).....	4	25	5	31	5	31	2	12	0	—
Farm (36).....	5	14	5	14	9	25	12	33	5	14
Non-depilated (22).....	3	13.5	3	13.5	6	27	10	45.5	0	0
Depilated (28).....	5	18	7	25	7	25	4	14	5	18

of the breeding room animals was free of infection during the entire period; while 14 per cent from the farm never showed *Bact. lepisepticum.*

(*d*) *Diet.*—A comparison of the two diet groups is shown in Table I and Text-figs. 6 and 7. Text-fig. 6 shows, with one exception, that throughout the entire period of observation the percentage of *Bact. lepisepticum* carriers among the "incomplete" diet series was higher than that of the group receiving the "complete" ration. However, there was no striking difference in the percentage of chronic snuffles cases, nor in the mortality rates. Therefore, it is difficult to interpret the effect of omitting fresh vegetables from the rabbits' diet for a period of 2 years, further than to state that no deleterious effect on general health could be observed. However, the fact that these

animals were more prone to carry *Bact. lepisepticum* in their nasal passages is of interest from an epidemiological point of view. Differences in host susceptibility, too slight to be recognized clinically, may apparently be determined by the more delicate bacteriological test.



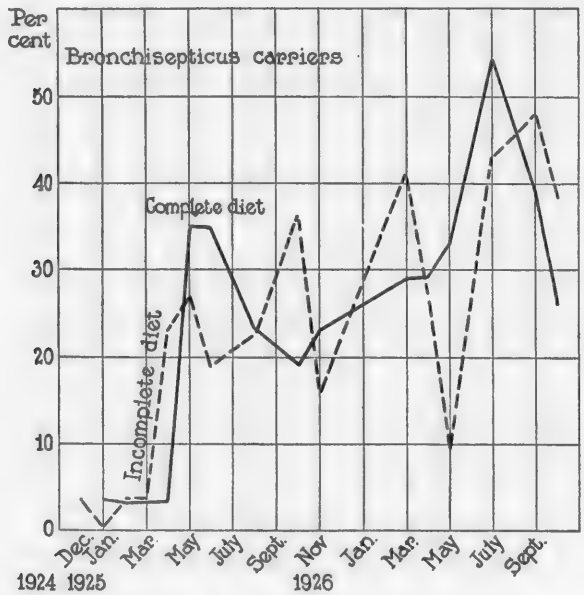
TEXT-FIG. 6. Spread of *Bact. lepisepticum* infection among the "complete" and "incomplete" diet groups.

And this modification of diet, by rendering the individual susceptible to mild infection, whereby he becomes a chronic carrier, may indeed be one influence which, by decreasing host resistance, raises dosage of bacteria available to the population to a dangerously high level.

Text-fig. 7 compares the percentages of *bronchisepticus* carriers in

the "complete" and "incomplete" diet series. No significant differences are apparent.

(*e*) *Season.*—From the figures available in this experiment, it is difficult to determine any effect of seasonal factors. During the summer of 1925 the percentage of *Bact. lepisepticum* carriers remained moderately low, during the winter of 1926, it rose to a great height,



TEXT-FIG. 7. Frequency of *B. bronchisepticus* carriers among the "complete" and "incomplete" diet groups.

and during the following summer declined. However, the complicating factors of artificial temperature changes and depilation make analysis of environmental influences impracticable.

(*f*) *Depilation.*—The same may be said of the artificial measures of depilation. This was carried out at about the same time that the temperature fluctuations were carried out and was followed by a sharp rise in carrier rate and appearance of chronic snuffles and definite

mortality from *Bact. lepisepticum* septicemia and pneumonia.[1] Probably the three factors, namely, exposure, temperature fluctuation, and seasonal influences were together responsible for the great increase in the prevalence and severity of the disease.

(*g*) *Behavior of Individual Rabbits.*—Throughout the 2 year period of observation certain animals appeared to be more resistant than others to *Bact. lepisepticum* infection. Hence, at the end of the experiment a final test was employed to emphasize these differences. For this purpose the Rivers D strain of *Bact. lepisepticum* was instilled into the nares of each rabbit. The reaction was slight; two or three acute cases of clinical snuffles developed, but in general the organisms tended to disappear rapidly from the nasal passages (1, *e*). Evidently continual exposure to infection over a period of 2 years had exerted a selective action in weeding out the less resistant individuals, thus leaving a group relatively resistant and showing little difference in response to this organism.

At the end of the experiment the 52 animals were grouped arbitrarily, according to their behavior during the 2 year period (Table III). Those animals that died composed the first group; those that reacted locally with chronic *Bact. lepisepticum* snuffles, the second; those that became chronic carriers of *Bact. lepisepticum*, the third; intermittent carriers, fourth; and finally, those that failed at any time to show *Bact. lepisepticum* in their nasal passages, fifth. Nine animals were placed in the first group. Four of these died from *Bact. lepisepticum* infection and showed in their lungs and hearts' blood the specific organisms. Ten showed symptoms of chronic snuffles and revealed at autopsy an extensive erosion of the nasal turbinates, with accumulations of pus in the sinuses. Nine of them showed pus in one or both middle ears. The mucoid form of *Bact. lepisepticum* was cultured from these lesions. Besides, from the nasal passages of six, ten to twenty colonies of the experimentally introduced Rivers D strain were found. Fourteen animals were chronic carriers. At autopsy two showed pus in the nasal passages and seven unilateral or bilateral otitis media. The mucoid form of *Bact. lepisepticum* was cultured from all of the ear

[1] Four of the six animals dying between December, 1925, and May, 1926, were depilated; on the other hand, a comparison of the reactions of the two groups as a whole (Table II) shows no notable differences.

TABLE

Summary of Fate of 52

No.	Fate	Clinical condition	Autopsy findings		
			Nasal passages	Ears	Lungs
E 54 B	Died 8/15/25	Torticollis	Normal	Pus, bilateral	Normal
E 83 F–S	" 12/ 7/25		"	Normal	Congestion, hemorrhage
E 16 B–S	" 12/12/25	Diarrhea; emaciated	Pus	"	Pleuro-pneumonia
D 85 F–S	" 1/ 8/26	Pregnant	"	"	Congestion
E 36 B	" 1/13/26	Snuffles; emaciated	"	"	Congestion consolidation
E 56 B–S	" 3/ 8/26	Snuffles; diarrhea	"	"	Hemorrhagic consolidation
C 69 F	" 5/21/26	Good	No pus	"	Hemorrhagic
E 90 F	" 9/16/26	Emaciation; torticollis	" "	Pus, bilateral	Normal
C 37 F–S	" 11/25/26	Cellulitis	" "	No pus	"
E 55 B–S	Killed 12/18/26	Emaciated; snuffles	Erosion; pus	Pus bilateral,	"
E 43 B–S	" "	Good	" "	" "	"
C 38 F–S	" "	"	Normal	Normal	"
E 57 B	" "	"	Erosion; pus	Pus, bilateral	"
E 93 B–S	" "	"	" "	" left	"
C 30 F–S	" "	"	" "	" bilateral	"
C 95 F	" "	"	" "	" left	"
A 85 F–S	" "	"	" "	" right	"
E 63 B–S	" "	"	" "	" bilateral	"
E 87 F	" "	"	" "	" "	"
81 F	" 12/20/26	"	Pus	" "	"
E 60 B	" "	"	"	Normal	"
E 18 F	" "	"	Normal	Pus bilateral	"
C 68 F	" "	"	"	" "	"
E 58 B–S	" "	"	"	" right	"
E 41 F–S	" "	"	"	" left	"
D 92 B–S	" "	"	"	" "	"
C 83 F–S	" "	"	"	" "	"
F 00 F–S	" "	"	"	Normal	"
E 44 B–S	" "	"	"	"	"
E 12 B–S	" "	"	"	"	"

III.

Experimental Rabbits.

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
Sterile	No *Bact. lep.*	*B. coli*	Sterile	Deaths
B. coli	" " "	Sterile	*B. coli*	"
Bact. lep. M	*Bact. lep.* M	"	*Bact. lep.* M	"
B. coli	" " "	"	*B. coli*	"
Sterile	*B. bronch. Bact. lep.* M	"	*Bact. lep.* M	"
Bact. lep. M	*Bact. lep.* M	"	" " "	"
" " "	*B. bronch. Bact. lep.* M	"	" " "	"
Sterile	No *Bact. lep.* M	—	—	"
"	*B. bronch.*	—	—	"
—	*Bact. lep.* D ++ M Inf.	*Bact. lep. M*	—	Chronic snuffles
—	" " " ++ " "	" " "	—	" "
—	" " " ++	—	—	" "
—	*B. bronch.* Inf. *Bact. lep.* M Inf.	*Bact. lep.* M	—	" "
—	" "	" " "	—	" "
—	*Bact. lep.* D ++ M Inf.	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " M	" " "	—	" "
—	" " "	" " "	—	" "
—	" " "	" " "	—	Chronic carriers
—	" " "	—	—	" "
—	" " D	*Bact. lep.* M	—	" "
—	" " "	" " "	—	" "
—	" " " + M Inf.	" " "	—	" "
—	" " " Inf.*bronch.*++	" " "	—	" "
—	*Bact. lep.* D+	" " "	—	" "
—	" " " +	" " "	—	" "
—	" " " G+	—	—	" "
—	" " D+++	—	—	" "
—	" " " ++	*Bact. lep.* M	—	" "

TABLE III

No.	Fate	Clinical condition	Autopsy findings		
			Nasal passages	Ears	Lungs
C 99 F	Killed 12/21/26	Good	Normal	Normal	Normal
C 49 F	" "	"	"	"	"
C 79 F	" "	"	"	"	"
E 65 B	" 12/22/26	"	Pus	"	"
C 44 F–S	" "	"	"	Pus, left	"
C 21 F	" "	"	"	Normal	"
C 91 F	" "	"	Normal	Pus, bilateral	"
D 62 F–S	" "	"	"	" left	"
E 69 F–S	" "	"	"	" "	"
E 77 F	" "	"	"	" "	"
C 46 F	" "	"	"	Normal	"
C 75 F–S	" "	"	"	"	"
D 44 F	" "	"	"	"	"
E 45 F	" "	"	"	"	"
E 75 B	" "	"	"	"	"
E 35 F	" "	"	"	"	"
E 28 F	Reserved	"	—	—	—
C 67 F–S	Killed 12/23/26	"	Normal	Pus, bilateral	Normal
C 92 F–S	" "	"	"	Normal	"
C 29 F–S	" "	"	"	"	"
E 97 F–S	" "	"	"	"	"
E 25 F–S	Reserved	"	—	—	—

Bact. lep. D = Rivers D type, instilled artificially 12/1/26.
Bact. lep. M = Mucoid type, spontaneous infection.
Bact. lep. G = Variant type, from Rivers D.
Inf. = Many colonies.

—*Concluded.*

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
–	*Bact. lep.* D+++	–	–	Chronic carriers
–	" " "++ G++	–	–	" "
–	" " M	–	–	" "
–	" " D+	–	–	Intermittent carriers
–	" " M Inf.	*Bact. lep.* M	–	"
–	" " D Inf.	–	–	"
–	*B. bronch.*	*Bact. lep.* M	–	"
–	D + *B. bronch.* Inf.	" " "	–	"
–	"++	D Inf.	–	"
–	*B. bronch.*	*Bact. lep.* M	–	"
–	*B. bronch.* Inf. *Bact. lep.* D+	–	–	"
–	*Bact. lep.* D+	–	–	"
–	*B. bronch.* Inf. *Bact. lep.* D+	–	–	"
–	*Bact. lep.* D Inf.	–	–	"
–	" " " "	–	–	"
–	*B. bronch.* Inf.	–	–	"
–	–	–	–	"
–	*Bact. lep.* D++	*Bact. lep.* M	–	Free
–	" " Inf.	–	–	"
–	*B. bronch.* Inf. *Bact. lep.* D+	–	–	"
–	" " " +	–	–	"
–	–	–	–	"

lesions and from the nasal passages of four. The Rivers D strain of *Bact. lepisepticum* was cultured from the nasal passages of eleven. Fourteen of the animals were classed as intermittent carriers. At autopsy pus was found in the nasal passages of three and in the middle ears of five. The mucoid form of *Bact. lepisepticum* was recovered from the ears of three; the D form from one. From the nasal passages of one mucoid colonies of *Bact. lepisepticum* were obtained; from nine, D forms. Five animals showed no *Bact. lepisepticum* in their nasal passages at any time during the experiment. At autopsy the nasal passages of all appeared normal. Pus was found in the middle ear of one. From here the mucoid form of *Bact. lepisepticum* was cultured. All four showed a few colonies of the Rivers D form.

When the animals are placed in these arbitrary groups, their differences in behavior appear the more striking. That some animals remained clinically healthy and free of infecting organisms during summer and winter, and at the time when temperature fluctuations and exposure were extreme, cannot be explained by chance. They were scattered amongst their companions, many of which were carriers, some snuffles cases, and still others ill with pneumonia. It seems more reasonable to regard the differences in behavior of these animals as due to innate and non-specific differences in their resistance.

Virulence.—At various times during the experiment, virulence titrations were made on strains of *Bact. lepisepticum* recovered from the nasal passages of various individual rabbits in the room. The technique of these tests has been discussed elsewhere, and the results of a number of them recorded in detail. The critical titrations were made by instilling equal doses of the various cultures into the nasal passages of young rabbits of similar age and weight, obtained from the breeding room. Subsequently, with these results as a basis, the mouse titration method was employed. This technique, although far more artificial, and of little or no value unless controlled by "natural" virulence titration, enabled us to examine a greater number of cultures and at more frequent intervals.

Many of these tests have been described previously.[2] In one instance[3] the results of intranasal titrations in rabbits of Cultures 544

[2] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362, 366–376.

[3] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362.

from Rabbit E 44 and 329 from Rabbit C 29 are summarized. In Tables IV, V, and VI the results of the intraperitoneal mouse titrations are shown. Culture 590 came from Rabbit E 90; Culture 560 was obtained on three different occasions from Rabbit E 60. Thus the strains in these tables may be identified: those in the 500's came from E rabbits; in the 400's from D rabbits; in the 300's from C rabbits, etc. These titrations emphasize two facts; first, that the form of *Bact. lepisepticum* brought into the room by the attendant and becoming endemic in the population was of the mucoid variety, similar to that found at the rabbit farm in New City, at Saranac, New York, and in other rabbits at the Rockefeller Institute; and secondly, that the virulence of all of these cultures proved equal and of moderate degree.

General Considerations.—The analysis of the part played by certain factors in the spread of *Bact. lepisepticum* throughout this population is rendered easier by reason of the control of disturbing influences. All the animals were exposed equally to the chance of infection so that theoretically those in the "complete" diet group should have been slightly more resistant than those receiving the "incomplete" ration. We assume that none of the rabbits had been previously exposed to the infection, and that none of the infecting organisms was in the room at the beginning of the experiment. We know that the infecting potentialities or virulence of the various strains of *Bact. lepisepticum* remained uniform throughout the entire period, and that the source of infection—attendants carrying organisms from infected rabbits in the neighboring room—remained constant.

At the outset, foci of infection were established in the nasal passages or middle ears of the rabbits naturally more susceptible, or rendered so by dietary deficiencies. During the succeeding 6 months available dosage was maintained at a level by chance contact with the attendant and by spread from local foci. Throughout the winter of 1925–26, procedures similar but more drastic than natural conditions were carried out to decrease further the resistance of the population. Dosage (carrier rate) increased rapidly, the weaker individuals succumbed to *Bact. lepisepticum* pneumonia and septicemia, and others developed local otitis media and snuffles. Still others, exposed to infection, remained refractory. As summer came on and the tem-

perature fluctuations were less severe, the population, reduced in numbers by the selective weeding our process, became more resistant, dosage fell, and cases of snuffles and numbers of deaths became less frequent.

In this experiment, therefore, factors influencing host susceptibility influenced the dosage of available bacteria and thereby determined the amount and severity of infection ensuing.

Control of Bact. lepisepticum Infection. Description of a Successfully Operated Rabbit Breeding Room.

As stated in the introduction, the purpose of this experiment was to test the conclusions derived from our studies of rabbit snuffles and pneumonia by attempting to keep a population of rabbits free of *Bact. lepisepticum* infection. The various procedures in this experiment were governed by two principles: first, the maintenance of host resistance at a maximum, and second, the decreasing of available dosage of *Bact. lepisepticum* to a minimum.

A special room was chosen in the midst of the space used for our normal rabbit stock. The room measures about 15×30 feet and has accommodations for thirty breeding females, ten males, and about fifty weaned young stock. Before bringing any animals into the room, the walls and floors were scraped carefully and washed with lysol, and the breeding cages were sterilized. The rabbits employed were selected from the normal stock obtained from various dealers within a radius of 200 miles of New York City. Such animals, upon their arrival at the Institute, were placed in isolation rooms, where they were observed for a period of 2 to 4 weeks. During this period any animals showing clinical evidence of snuffles were discarded.

During the spring and summer of 1923 several hundred of these animals were cultured from the nasal passages to detect the presence of *Bact. lepisepticum*. In view of the usual high percentage of carriers (1, *f*), a great many animals were examined before a suitable stock of breeders was obtained. No animal was admitted to the breeding room until three successive nasal cultures failed to demonstrate the presence of a single colony of *Bact. lepisepticum*. When an animal was finally admitted, its fur was sponged carefully with 5 per cent lysol. Thus by September a stock of about thirty females and ten males was accumulated.

The animals were cared for by an experienced attendant who has maintained a strict régime of cleanliness. In general, the door to the room is kept locked. Before entering the room, the attendant is careful to change his coat and wash his hands. The food is always taken from a fresh supply, and the animals are given a considerable amount of personal attention. The temperature in the room is regulated carefully at about 68°. The cages are cleaned every 2nd day.

The results of this experiment are striking, for by continuing the above careful methods of supervision we have maintained a breeding room entirely free of *Bact. lepisepticum* infection.[4]

At monthly intervals from September, 1923, to March, 1924, the entire breeding room was examined bacteriologically. Again, on September 24, 29, October 6, and December 17, each animal was cultured from the nasal passages. On March 18, 1925, twenty-two showed organisms of the *B. coli* group on hemolyzed blood agar plates streaked from cultures of the nasal passages. These organisms we have always considered as intestinal contaminants, occurring only at the external nares. Twelve showed *B. bronchisepticus*. These animals continued to be consistent carriers of this organism from the time they were selected for the breeding room until they died, or were discarded. None showed colonies of *Bact. lepisepticum*. On several occasions during 1926 young stock taken from this room have been cultured. On no occasion has *Bact. lepisepticum* been recovered. There have been no cases of pneumonia in the room, no clinical snuffles, no abscesses, and no otitis media.

The breeding record is summarized in Table IV. In it are tabulated: (1) the number of young weaned 2 months after birth; (2) the month of these weanings; (3) the number and date of sterile matings; (4) the date and number of times the young were destroyed; and (5) the fate of the various breeders. The males are not included in this

[4] A few of the animals are affected with ear canker. This is treated in its early stages by local applications of tincture of iodine and lanolin. Likewise there is a certain amount of pinworm infection present, which can only be demonstrated at autopsy.

In 1924 some of the animals showed at autopsy the pathological changes characteristic of rabbit encephalitis. Clinically, the animals appeared perfectly healthy. No further diseases have been found.

TABL

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.	May
1					3				4			5							7		
2			6			2			M.										4		
3											6						4				
4			4					3	D.												
5			x					2		4				4			6		M.		
6								2 M.	D.												
7				4					3					5					3	D.	
8					2							5				M.		x		M.	D.
9								3	D.												
10										4					3				5	Dis.	
11										5					x					4	
12										3					x				2		
13																4		x		x	
14														4	M.			2		M.	
15																	3		D.		
16																		5		x	
17																			3		
18																	5			1	
19																		6	D.		
20															6					2:x	
21															3				1		
22															x		x	M.		M.	
23															3		x				
24															7				7		
25																x				2	Di
26																				4	
27																			1		x
28																		x			
29																			1		
30																		M.			
31																x					4
32																		2			
33																			1		

D., signifies died; x, destroyed; Dis., discarded; M., sterile mating.

July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1927	Total
			6	D.															29
2	M.		Dis.																14
						4	Dis.												19
																			7
6			1	1			5						3	Dis.					32
																			2
																			15
																			7
																			3
																			12
	2	Dis.																	11
	1		1	Dis.															7
			3		x	Dis.													7
			1	Dis.															7
																			3
			2			3		M.			5	Dis.							15
		3		M.		M.	4					M.	M.	Dis.					10
	1				4				3		4			3					21
																			6
		3	D.																11
	6	x				8					5	Dis.							23
	1			2	Dis.														3
2					2					5		4			3				19
			5		M.				7	x				M.	Dis.				29
																			2
M.				4					6			7		M.	M.	M.	M.		24
M.					2	D.													3
M.	x				2	M.			6				2			4			16
				5					8			1		M.			3		22
x	Dis.																		4
																			4
1x				1			6	Dis.											10
			5				8												20

TABLE IV

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.
34																			6	
35																			D.	
36																			M.	
37																				
38																				
39																				
40																			x	
41																				1
42																			7	
43																				
44																				
45																				
46																				
47																				
48																				
49																				
50																				
51																				
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57																				
58																				
59																				
60																				
61																				
62																				
63																				
64																				
Total.....			10	4	5	2	0	10	7	16	6	10	0	13	22	4	18	15	48	14

ontinued.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1927	Total
	x				5					4		5					5			28
	x				5					4			3		M.	M.	Dis.			12
4	M.	D.																		4
				2			5	M.			6		M.		M.	D.				19
	M.				5				7			7		M.	M.	M.				25
		2		x			4	M.				1			4	M.				12
		5				4					4				2	M.	M.			15
		1	D.																	2
	2				5	Dis.														14
	x			M.	M.			7					2		M.	M.		M.		9
	x			x			7				3				3		M.	M.		13
						4				5		3		M.			5			17
			x		M.				7	M.	Dis.									7
								7			1		M.		M.	Dis.				8
							4			2	D.									6
							3	M.					2		x	M.	Dis.			5
							3				2		2			3				10
						x					7			5	M.	M.	M.			12
									4	M.			x			1		x		5
								5			M.					6				11
											2M.				3			2		7
												2			M.	M.				2
											2				1			6		9
											1				4		M.			5
																2				2
															4					4
															2					2
																		4		4
																	5			5
															2			4		6
																		4		4
38	13	19	6	26	33	18	41	42	18	45	33	32	21	10	28	15	19	23		701

table. The deaths recorded were due to parturition complications. None of these animals was infected with any microorganism. Animals were discarded for several reasons: first, for sterility or neglect of young, and secondly, for not conforming to the desired type. Dutch, Belgian, and albino crosses were specially selected because of their tendency to produce rapidly developing, small, heavy stock. During the year 1924, 95 young were weaned, an average of 8 per month and 3 per rabbit. In 1926, 265 were weaned, 22.2 per month, 9 per rabbit, and in 1926, 327 were weaned, 27.2 per month, 11 per rabbit.

The results of this experiment indicate that it is possible to maintain a stock of rabbits free from *Bact. lepisepticum* infection, although surrounded by groups in which the disease is widespread, by proper attention to the general condition or "natural" resistance of the animals, and by measures which minimize the available dosage of pathogenic microorganisms.

DISCUSSION AND SUMMARY.

In this paper we have attempted to describe the manner of spread of an endemic, native, respiratory infection and a method for its control. The essential factor determining the prevalence of such an endemic disease is, we believe, host susceptibility, which is controlled by hereditary and environmental influences. Furthermore, it seems probable that the amount of this population susceptibility determines the dosage of specific microbes available to the population.

An increase in dosage in the herd is followed by an increase in the spread and severity of the infection, and a decrease by a corresponding alleviation. Hence, two methods for the prevention of epidemics are available: (1) an enhancement of population resistance, and (2) the reduction to a minimum of available dosage. These procedures have proved successful for 3 years in maintaining a population of breeding rabbits, in the midst of a badly infected community, entirely free from *Bact. lepisepticum* infection.

Confirmation of the above conclusions has been gained from other studies in the field of experimental epidemiology. Dr. D. T. Smith (2), at Saranac, New York, found that changes in population susceptibility were responsible for a severe outbreak of *Bact. lepisepticum* infection and septicemia. Freund (3), at Berlin, has just published

an interesting account of respiratory epidemics of rabbits and guinea pigs, apparently brought about by sudden changes in temperature and housing conditions. Pneumonia and Pasteurella infection, endemic in the population, increased suddenly in extent and severity. Nevertheless, neither endemic nor epidemic strains of the microorganisms were found to be especially virulent. Dr. Theobald Smith (4), in a study of paratyphoid epidemics of guinea pigs, has made similar observations. He noted that pregnant females acted as the foci of infection, and that from these individuals, presumably of lessened resistance, the bacteria were given off and infection was spread.

The studies in experimental epidemiology are rapidly reaching a stage where they may be applied to the problems of human disease. Indeed, more recent observations of the mode of spread of pneumonia (5–7), scarlet fever (8), typhoid (9, 10), plague (11), diphtheria (12–14), measles (15), and tuberculosis (16–18) increasingly show a tendency to discard the theory of fluctuating microbic virulence and to emphasize the importance of the host factors.

BIBLIOGRAPHY.

1. Webster, L. T., *et al.*, *J. Exp. Med.* (*a*), 1924, xxxix, 837, 843, 857; xl, 109; 1927, xlv, 529; (*b*) 1926, xliii, 555, 573; xliv, 343, 359; (*c*) 1924, xxxix, 857; 1926, xliii, 573; (*d*) 1926, xliv, 343; (*e*) 1926, xliii, 573; (*f*) 1924, xxxix, 843, 857.
2. Smith, D. T., *J. Exp. Med.*, 1927, xlv, 553.
3. Freund, R., *Z. Hyg. u. Infectionskrankh.*, 1926, cvi, 627.
4. Smith, T., *J. Exp. Med.*, 1927, xlv, 353, 365.
5. Chapin, W. E., *Ayer Clin. Lab. Bull. Penn. Hosp.*, No. 9, 1925.
6. Powell, J. P., Atwater, R. M., and Felton, L. D., *Am. J. Hyg.*, 1926, vi, 570.
7. Rosenau, M. J., Felton, L. D., and Atwater, R. M., *Am. J. Hyg.*, 1926, vi, 463.
8. Stevens, F. A., *J. Am. Med. Assn.*, 1926, lxxxvii, 2137; Blake, F. G., and Trask, J. D., *J. Clin. Inv.*, 1926, iii, 397.
9. Leach, U. S. *Pub. Health Rep.*, 1926, xli, 705.
10. Falk, I. S., *J. Prev. Med.*, 1926, i, 53.
11. Wu, L.-T., League of Nations Health Organization. A treatise on pneumonic plague, Geneva, 1926.
12. Deacon, W. J. V., *Michigan Dept. Health*, 1925, 25.
13. *New York City Dept. Health Weekly Bull.*, 1926, xv (November 6).
14. Dudley, S. F., *Med. Research Council, Rep. No. 111*, 1926.
15. Schütz, Fr., Die Epidemiologie der Masern, Jena, 1925.
16. Neufeld, F., *Deutsch. med. Woch.*, 1925, li, 341.
17. *Statistical Bull. Metropolitan Life Ins. Co.*, 1926, vii, 9, 1.
18. Krause, A. K., and Willis, H. S., *Am. Rev. Tuberc.*, 1926, xiii, 52.